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Triacylglycerol Lipases of the Yeast

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Mein besonderer Dank gilt…

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Summary

The yeast *Saccharomyces cerevisiae* is a widely used and reliable model organism to study lipid biology, because metabolic routes such as non-polar lipid biosynthesis, storage and turnover are well described and characterized. Lipid droplets are the storage compartment for non-polar lipids, triacylglycerols and steryl esters. Four enzymes contribute to non-polar lipid synthesis in *S. cerevisiae* namely the triacylglycerol synthases Dga1p and Lro1p and the steryl ester synthases Are1p and Are2p. These proteins are located to the endoplasmic reticulum, but Dga1p was also found in lipid droplets. In contrast, enzymes involved in cleavage of triacylglycerols are mainly located to lipid droplets. The major triacylglycerol lipases Tgl3p, Tgl4p and Tgl5p, however, do not only facilitate triacylglycerol degradation, but also contribute to the biosynthesis of phospholipids acting as lysophospholipid acyltransferases. Catabolic as well as anabolic pathways of non-polar lipids are well described in the yeast *S. cerevisiae,* but data about molecular regulatory mechanisms are still rare. Therefore, we studied regulation of the triacylglycerol lipases Tgl3p, Tgl4p and Tgl5p with emphasis on their multiple functions. One major finding was that the major triacylglycerol lipase Tgl3p is mainly regulated by substrate limitation. In the absence of sufficient substrate, Tgl3p is completely or partially restricted to the ER, where non-polar lipids are synthesized. Additionally, protein level and stability are strongly reduced under these conditions. Both catabolic and anabolic activities of Tgl3p seem to be restricted to its original compartment, the lipid droplets. In contrast, regulation of triacylglycerol lipases on the gene expression level is of only minor importance. Furthermore, this study reports a regulatory link between non-polar lipid degradation and synthesis in the yeast *S. cerevisiae*. A yeast strain lacking the major triacylglycerol lipases does not only accumulate triacylglycerols but also steryl esters. We showed that a block in triacylglycerol degradation and the resulting triacylglycerol accumulation leads to a shift in non-polar lipid synthesis. Whereas triacylglycerol formation is reduced, steryl ester biosynthesis is enhanced. Taken together, this study provides insight into the regulatory network of non-polar lipid metabolism in the yeast, which is important for lipid homeostasis in this microorganism.

Zusammenfassung

Die Hefe *Saccharomyces cerevisiae* ist ein weit verbreiteter und zuverlässiger Modellorganismus um den Fettstoffwechsel zu untersuchen, da Prozesse wie der Neutrallipid-Stoffwechsel gut beschrieben sind. Lipidpartikel sind der Speicherort für die Neutrallipide, Triglyceride und Sterolester. Vier Enzyme tragen zur Neutrallipidsynthese bei, nämlich die Triglycerid-Synthasen Dga1p und Lro1p, und die Sterolester-Synthasen Are1p und Are2p. Diese Proteine befinden sich im Endoplasmatische Retikulum, wobei Dga1p auch am Lipidpartikel gefunden wurde. Im Gegensatz dazu sind Enzyme die am Abbau der Triglyceride beteiligt sind hauptsächlich am Lipidpartikel zu finden. Die wichtigsten Triglycerid-Lipasen Tgl3p, Tgl4p und Tgl5p sind nicht nur am Triglycerid-Abbau beteiligt, sondern tragen auch zur Biosynthese von Phospholipiden bei, u. a. durch ihre lysoPhospholipid Acyltransferase-Aktivität. Abbau- sowie Synthesewege des Neutrallipid-Stoffwechsels sind in der Hefe *S. cerevisiae* gut beschrieben, aber Daten über molekulare Regulationsmechanismen fehlen. Deshalb haben wir die Regulation von Triglycerid-Lipasen Tgl3p, Tgl4p und Tgl5p mit Schwerpunkt auf deren duale Funktionen untersucht. Eine wichtige Erkenntnis war, dass die Triglycerid-Lipase Tgl3p hauptsächlich durch Substratlimitierung geregelt wird. In Abwesenheit von ausreichend Substrat wird Tgl3p ganz oder teilweise im Endoplasmatischen Retikulum zurückgehalten. Zusätzlich sind Proteinniveau und Proteinstabilität unter solchen Bedingungen stark reduziert. Katabole sowie anabole Aktivitäten von Tgl3p sind absolut auf dessen ursprüngliche Lokalisation, die Lipidpartikel beschränkt. Im Gegensatz dazu ist die Regulierung der Triglycerid-Lipasen auf Genexpressionsebene nur von geringer Bedeutung. Des Weiteren zeigt diese Studie eine regulatorische Verknüpfung zwischen Neutrallipid-Abbau und Synthese in der Hefe *S. cerevisiae*. Eine Mutante, der alle wichtigen Triglycerid-Lipasen fehlen, reichert nicht nur Triglyceride, sondern auch Sterolester an. Wir konnten zeigen, dass ein Blockieren des Triglycerid-Abbaus und die daraus resultierende Triglycerid-Anreicherung zu einer Verschiebung der Neutrallipid-Synthese führen. Während die Triglycerid-Bildung verringert wird, wird die Sterolester-Biosynthese induziert. Zusammenfassend bietet diese Studie einen Einblick in das regulatorische Netzwerk des Neutrallipid-Stoffwechsels in der Hefe, das für die Aufrechterhaltung der Lipidhomöostase in diesem Mikroorganismus von großer Bedeutung ist.

Aim and Hypothesis

Tgl3p, Tgl4p and Tgl5p are the major triacylglycerol (TG) lipases of the yeast *Saccharomyces cerevisiae* (Athenstaedt & Daum 2005, 2003; Kurat et al. 2006). These hydrolytic enzymes were demonstrated to be located to lipid droplets (LD) where they facilitate degradation and turnover of TG. The present work focuses on storage and especially on mobilization of TG in the yeast *S. cerevisiae*, a highly suitable and well established model organism for studies of lipid biology and biochemistry. Different experiments from our laboratory revealed a dual function of these enzymes (Rajakumari & Daum 2010a, 2010b; Rajakumari et al. 2010). Tgl3p, Tgl4p and Tgl5p do not only act as hydrolytic enzymes but also contribute to phospholipid metabolism as lysophospholipid acyltransferases. It has been shown that these activities work completely independently from each other because the three TG lipases contain distinct and independent active centres for lipolysis and acyltransferase reactions. This finding has opened a novel view of these enzymes with respect to regulation.

The aim of this PhD thesis was to shed light on the cellular and molecular regulation of the three major TG lipases from the yeast. More precisely, we wanted to elucidate how TG lipases channel products of hydrolysis to the synthesis of complex lipids by addressing following questions:

(i) How do TG lipases react on TG formation/depletion under different conditions, e.g. under substrate limitation?
(ii) How does the presence/absence of lipid droplets influence the subcellular localization of TG lipases?
(iii) Does a feedback regulation exist from TG lipolysis to the synthesis of the non-polar lipids TG and SE?

By addressing the questions raised above we proposed to shed light on the regulatory network of non-polar lipid metabolism in the yeast.

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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*

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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

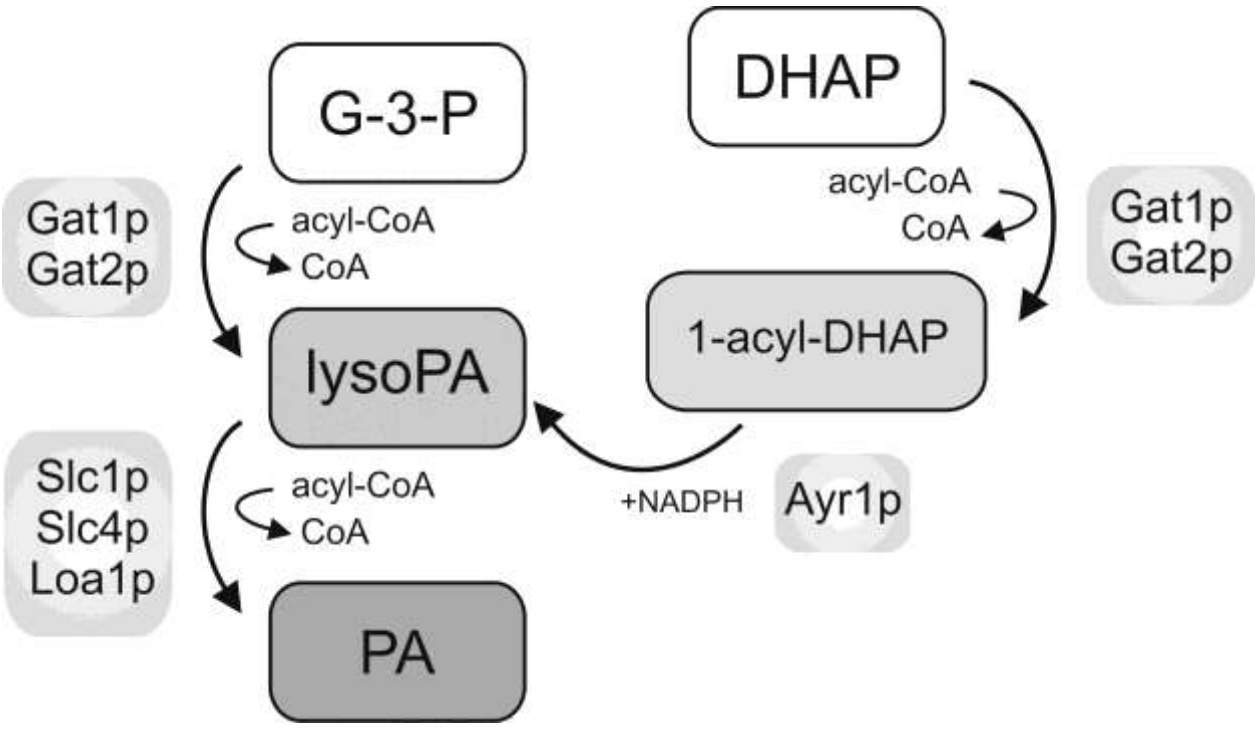

Fig. 1 Formation of phosphatidic acid in the yeast *S. cerevisiae.* LysoPA can be either formed through the G-3-P or the DHAP pathway. Both reactions require acylation of the respective precursors by Gat1p or Gat2p, respectively. 1-Acyl-DHAP needs to be reduced to lysoPA by Ayr1p in an NADPH dependent 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

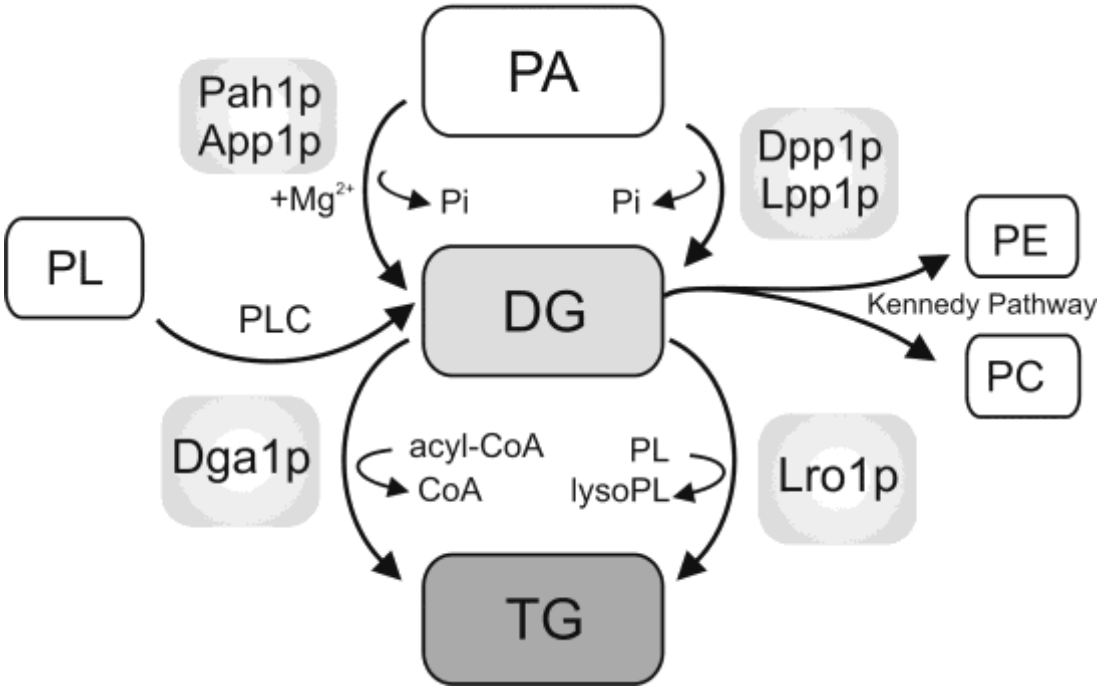

Fig. 2 Formation of diacylglycerol and triacylglycerol in the yeast *S. cerevisiae.* Formation of DG by dephosphorylation of PA is catalyzed by Pah1p and App1p in an Mg^{2+} dependent, or by Dpp1p and Lpp1p in an Mg^{2+} independent reaction. DG is then acylated to form TG by either an acyl-CoA dependent 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

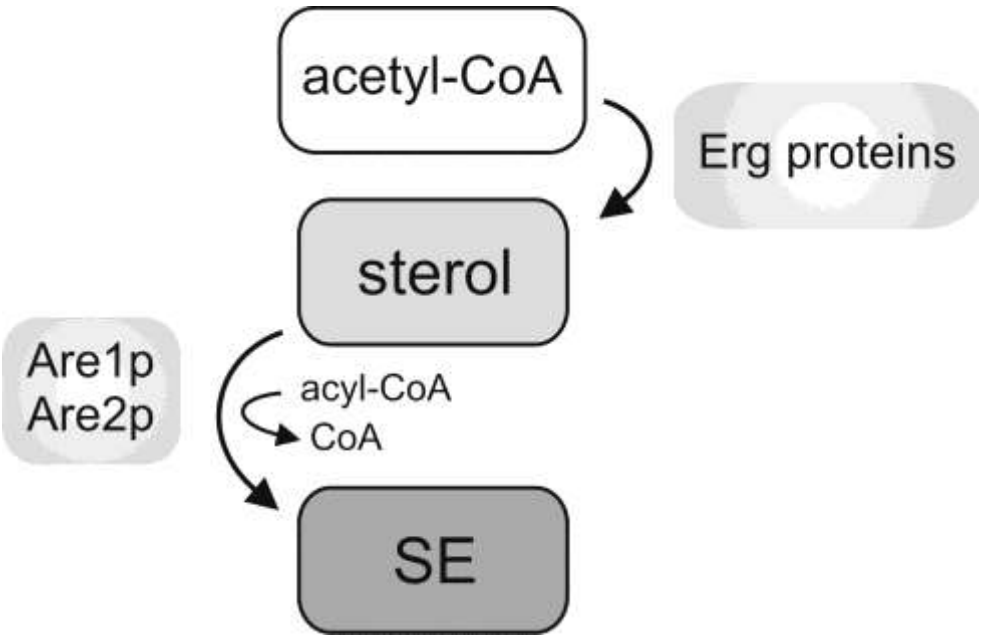

Fig. 3 Formation of sterols and steryl esters in the yeast *S. cerevisiae*. Sterols are formed from their very first precursors acetyl-CoA through several catalytic steps catalyzed by Erg proteins. Sterols are then further acylated in an acyl-CoA dependent reaction performed by Are1p or Are2p, respectively.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

FIGURE 4

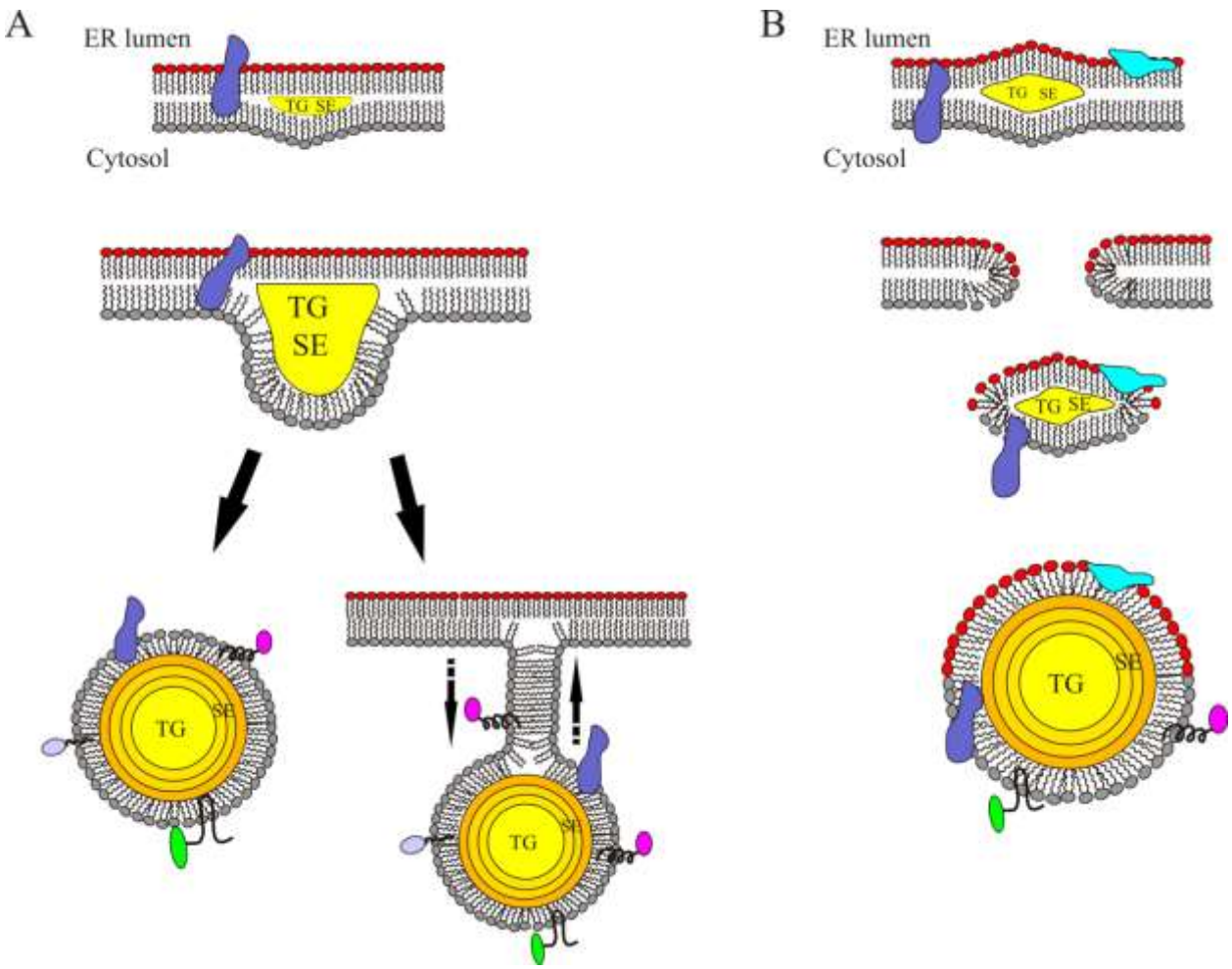

Fig. 4 Models of lipid droplet biogenesis. A: TG accumulates between the two leaflets of the ER membrane. After reaching a critical size, the growing LD buds off the ER membrane. Continuities between the ER membrane and the LD might deliver LD proteins from the ER to their destination. According to this 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

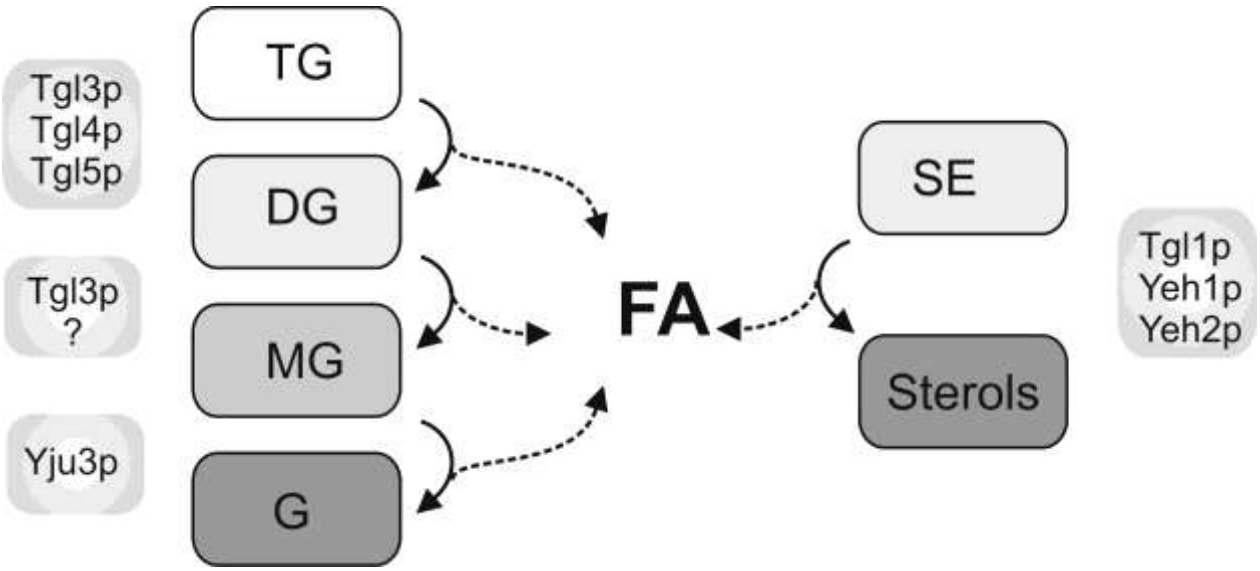

Fig. 5 Mobilization of triacylglycerols and steryl esters in the yeast *S. cerevisiae.* TG is degraded in a cascade of hydrolytic reactions. Tgl3p, Tgl4p and Tgl5p catalyze the hydrolysis of TG to DG and FA. Tgl3p is also a DG lipase. Other DG lipases of the yeast have not yet been identified. Yju3p 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.

CHAPTER II

Analysis of Yeast Lipid Droplet Proteome and Lipidome

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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.

[Figure 1 here]

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.

[Figure 2 here]

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.

[Figure 3 here]

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.

[Figure 4 here]

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).

[Table 1 here]

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

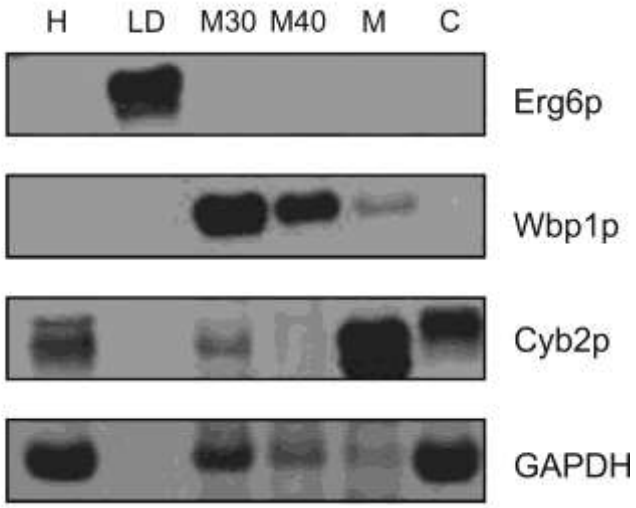

Fig. 1 Quality control of lipid droplets isolated from *S. cerevisiae.* Cells were grown to the stationary phase on glucose medium. Western blot analysis of 10 µg protein from each fraction was performed as described in the Methods section. Homogenate (H), LD (LD), 30,000xg microsomes (M30), 40,000xg 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

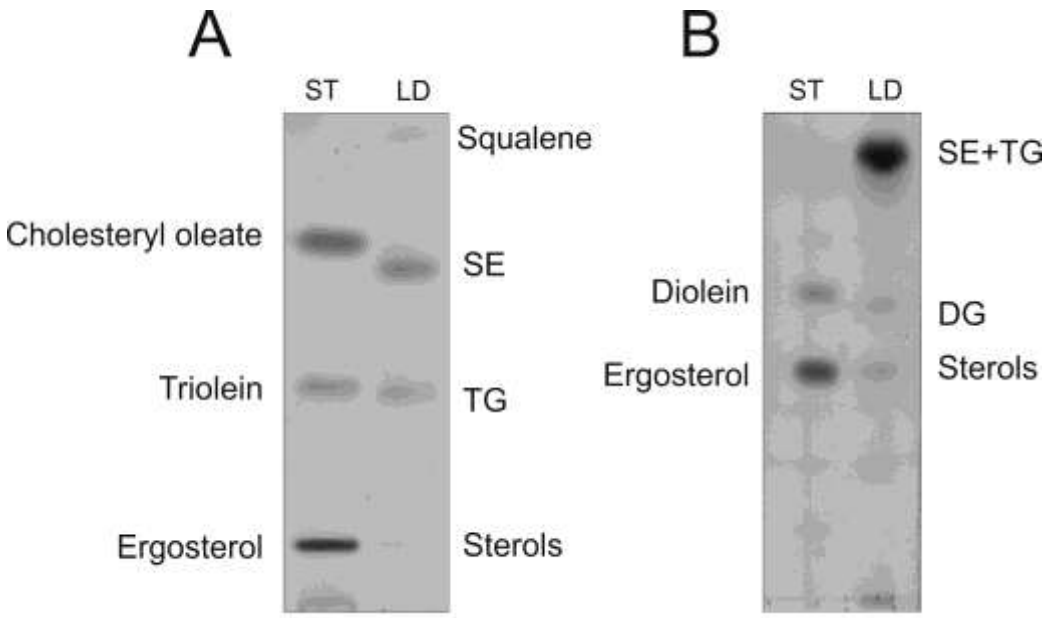

Fig. 2 Non-polar lipid analysis of lipid droplets from *S. cerevisiae.* For the TLC analysis of non-polar lipids different solvent systems were used (see Methods section). Cells were grown to the stationary phase on glucose medium. Lipids were extracted and separated using light petroleum/diethyl ether/acetic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

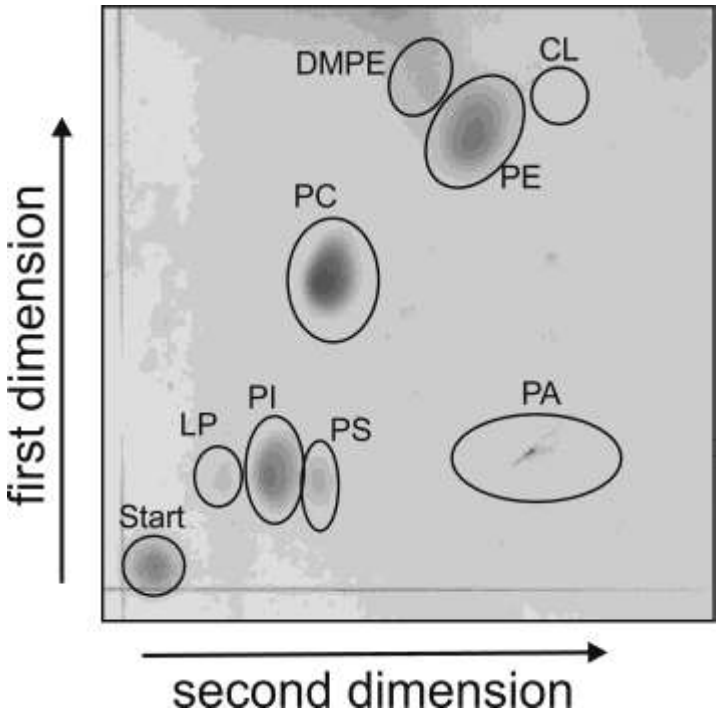

Fig. 3 Phospholipid analysis of lipid droplets from *S. cerevisiae.* Two dimensional TLC of individual phospholipids from *S. cerevisiae* LD was performed as described in the Methods section. Cells were grown to the stationary phase on glucose medium. Lipids were extracted and separated using chloroform/methanol/25%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

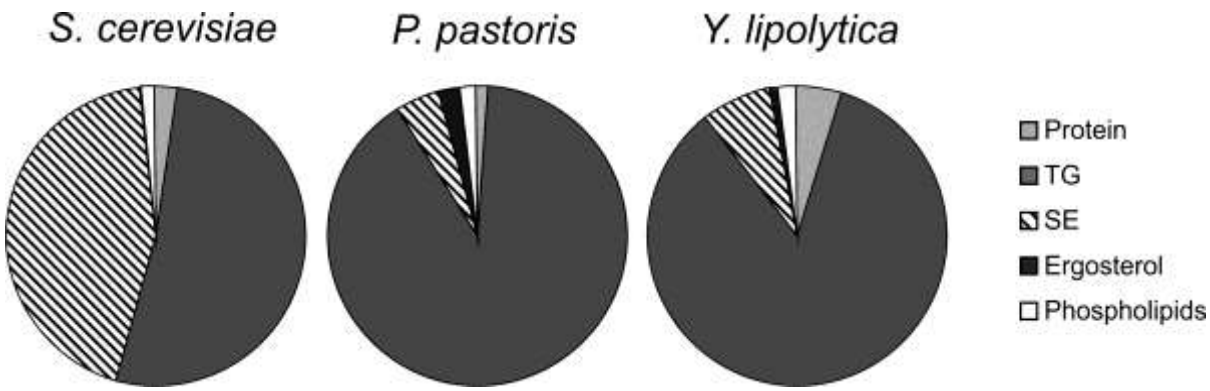

Fig. 4 Major components of lipid droplets from *S. cerevisiae, P. pastoris* and *Y. lipolytica.* Data were obtained from Athenstaedt et al. (2006); Ivashov et al. (2012) and Leber et al., (1994). Relative amounts of TG (triacylglycerols), SE (steryl esters), ergosterol, phospholipids and protein were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

CHAPTER III

Regulation of the yeast triacylglycerol lipase Tgl3p by formation of non-polar lipids

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Abstract

Tgl3p, the major triacylglycerol lipase of the yeast *Saccharomyces cerevisiae*, is a component of lipid droplets, but also present in the endoplasmic reticulum at a minor amount. Recently, it was shown that this enzyme can also serve as a lysophospholipid acyltransferase (Rajakumari et al., 2010, *Mol. Biol. Cell* 21, 501-510). Here, we describe effects of presence/absence of triacylglycerols and lipid droplets on the functionality of Tgl3p. In a *dga1*Δ*lro1*Δ*are1*Δ*are2*Δ quadruple mutant lacking all four triacylglycerol and steryl ester synthesizing acyltransferases and consequently lipid droplets the gene expression of *TGL3* was only slightly altered. In contrast, protein level and stability of Tgl3p were markedly reduced in the absence of lipid droplets. Under these conditions the enzyme was localized to the endoplasmic reticulum. Even the lack of the substrate, triacylglycerol, affected stability and localization of Tgl3p to some extent. Interestingly, Tgl3p present in the endoplasmic reticulum seems to lack lipolytic as well as acyltransferase activity as shown by enzymatic analysis and lipid profiling. Thus, we propose that the activity of Tgl3p is restricted to lipid droplets, whereas the endoplasmic reticulum may serve as a parking lot for this enzyme.

Introduction

All types of eukaryotes including the yeast *Saccharomyces cerevisiae* contain lipid droplets (LD) (1–3). These organelles, also known as lipid particles or oil bodies, serve as a storage compartment for non-polar lipids. In *S. cerevisiae*, triacylglycerols (TG) and steryl esters (SE) are the two major classes of non-polar lipids. These components provide a source of energy, but also serve as important depots of building blocks for the formation of membrane phospholipids. The structure of LD is largely conserved in all eukaryotes (1). In the yeast, LD contain a hydrophobic core of TG and SE, approximately 50% each, which is surrounded by a phospholipid monolayer with a small but distinct set of proteins embedded (4–6).

The biogenesis of LD is still a matter of dispute. It is generally accepted that LD are derived from the endoplasmic reticulum (ER) where the majority of non-polar lipids are synthesized (7, 8). In various models which describe the formation of LD (for a recent review see (9)) TG and SE accumulate in the ER, are enwrapped by a phospholipid monolayer and leave the ER when reaching a critical size. LD are dynamic organelles and remain functionally and physically connected to the ER (10).

During the last few years, many enzymes involved in the formation of non-polar lipids have been identified and characterized. In the yeast *S. cerevisiae,* four acyltransferases contribute to non-polar lipid synthesis, namely Lro1p, Dga1p, Are1p and Are2p (11). At an early growth stage, TG are mainly formed by the phospholipid:diacylglycerol acyltransferase Lro1p which is exclusively localized to the ER (12, 13). The acyl-CoA independent enzyme requires a phospholipid, preferentially phosphatidylethanolamine or phosphatidylcholine, as an acyl-donor and resembles the human lecithin:cholesterol acyltransferase (LCAT). Alternatively, TG is formed by the acyl-CoA:diacylglycerol acyltransferase Dga1p, which is dually located to LD and the ER (14, 15). Dga1p uses activated fatty acids as a co-substrate and is the major TG synthase in *S. cerevisiae* grown under standard conditions, especially when cells reach the stationary phase (16). SE of *S. cerevisiae* are synthesized by two acyl-CoA:sterol acyltransferases named Are1p and Are2p (17), two closely related enzymes located to the ER. Are1p and Are2p exhibit slight differences in their substrate specificities (18). The major SE synthase Are2p preferentially utilizes ergosterol as a substrate, whereas Are1p esterifies ergosterol precursors, mainly lanosterol, as well. Additionally, the two SE synthases contribute to TG synthesis although with minor efficiency (16). A yeast strain lacking all four non-polar lipid synthesizing enzymes, the *dga1*Δ*lro1*Δ*are1*Δ*are2*Δ quadruple mutant (QM),

is still viable under standard growth conditions but does not form LD (16). In this mutant, several LD proteins are retained in the ER (19).

Mobilization of TG from LD requires hydrolytic enzymes located to the surface monolayer membrane of LD (20–22). The three yeast TG lipases identified so far, Tgl3p, Tgl4p and Tgl5p catalyze hydrolysis of TG to diacylglycerols (DG) and free fatty acids. Interestingly, previous studies from our laboratory described additional functions of these enzymes as lysophospholipid acyltransferases with preferences for either lysophosphatidylethanolamine or lysophosphatidic acid, respectively, as substrates (23–25). In *tgl3*Δ and *tgl5*Δ strains the amounts of total phospholipids are reduced supporting the view that acyltransferase activities of Tgl3p and Tgl5p are also relevant *in vivo*. Beside the prominent function as a TG lipase, Tgl4p acts as a lysophospholipid acyltransferase but also exhibits phospholipase and SE hydrolase activities *in vitro*. These findings opened a novel view of these enzymes and also possible additional regulatory aspects which might contribute to their catabolic and anabolic role in lipid metabolism.

The present study is focused on the regulation of the major TG lipase Tgl3p. We investigated consequences of a lack of non-polar lipids on the expression and protein level, the enzymatic functions and the subcellular distribution of the enzyme. We demonstrate that especially subcellular localization and stability of Tgl3p are dramatically affected by the formation of non-polar lipids. Effects of Tgl3p regulation on lipid metabolic network are discussed.

Materials and Methods

Strains and culture conditions- Yeast strains used in this study are listed in Table 1. Cells were grown aerobically either to the logarithmic or to the stationary growth phase at 30°C in YPD media containing 1% yeast extract, 2% glucose and 2% peptone. Yeast strains bearing plasmids were cultivated in synthetic minimal medium containing 0.67% yeast nitrogen base (U.S. Biochemical Corp.), 2% glucose and the respective amino acid supplements. Growth was monitored by measuring optical density at 600 nm (OD_{600}). Gal1 promoter-controlled genes were induced by growing cells in synthetic minimal medium containing 2% galactose as a carbon source.

Genetic techniques- Chromosomal tagging and deletions were generated by homologous recombination using the PCR-mediated method described by Longtine et al. (26). In brief, the

inserts for the construction of PGal1-GFP-Tgl3, Tgl3-Myc or *tgl3*Δ strains were obtained by PCR from plasmids pFA6a-HIS3MX6-PGAL1-GFP(S65T), pFA6a-13Myc-HIS3MX6, pFA6a-HIS3MX6 or pFA6a-URA3KL from *K. lactis*. Primers used for amplification of the respective DNA-fragments are listed in Table 2. 400-700 ng DNA were used for transformation of yeast strains employing the high-efficiency lithium acetate transformation protocol (27). After transformation, cells were plated on minimal medium lacking the respective amino acid for selection and incubated for 2 to 3 days at 30°C. Positive transformants were verified for correct integration of the fusion cassette by analytical PCR of whole yeast cell extracts.

For the construction of the pYES-Sec61-mCherry plasmid, the open reading frame of Sec61 lacking the stop codon was fused with the open reading frame encoding mCherry by overlap PCR. The respective templates were genomic DNA from *S. cerevisiae* and plasmid pUG36-mCherry. Prior to ligation, the insert and the vector were cleaved by *BamH*I and *Xho*I. Primers used for fusion PCR are listed in Table 2.

For episomal expression, *TGL3* including its promoter and terminator region was inserted into plasmid pRS315. Insertion cassettes were obtained by PCR using genomic DNA from *S. cerevisiae*. The promotor region of *TGL3* was inserted by cleavage with *Not*I and *BamH*I, the terminator region by cleavage of *Pst*I and *Hind*III. Open reading frames of $TGL3^{wild\ type}$ (wild type), $TGL3^{S237A}$ (mutation in lipase motif), $TGL3^{H298A}$ (mutation in acyltransferase motif) or $TGL3^{S237A;H298A}$ (mutations in both motifs) were inserted into the plasmid pRS315 containing promoter and terminator region by cleavage with *BamH*I and *Pst*I. Gene mutations were described previously by Rajakumari et al. (25). The plasmids were transformed into *tgl3*Δ. Primers used for cloning the respective open reading frame with sequences for HA-tag on the N-terminus and Myc-tag on the C-terminus of *TGL3* into pRS315 are listed in Table 2.

Isolation and characterization of subcellular fractions- Highly purified LD and microsomes were isolated from cells grown to the stationary phase following published procedures (4, 28, 29). The protein concentration of isolated fractions was analyzed by the method of Lowry et al. (30) using bovine serum albumin as a standard. Prior to protein analysis, samples of 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 10% and solubilized in 100 µL 0.1% SDS, 0.1 M NaOH.

SDS-polyacrylamide gel electrophoresis was carried out by the method of Laemmli (31) using 12.5% separation gels, and Western blot analysis was performed as described by Haid and Suissa (32). Proteins were detected by using rabbit or mouse antisera as the first antibody and peroxidase-conjugated goat anti-rabbit or anti-mouse IgG as second antibody. Primary antibodies were directed against the Myc-tag/HA-tag, Wbp1p (ER-marker), GAPDH (cytosolic marker), Ayr1p, Erg6 and Erg1p (LD markers). 10 µg of each fraction were loaded onto SDS-gels for Western blot analysis. Comparative immunoblot data were from the same blot. Relative intensities of Western blots were calculated using ImageJ program.

Preparation of total cell extract for lipid analysis- Total cell extract for lipid analyses was prepared by growing yeast cells to the stationary phase. Cells were harvested by centrifugation at 3,000xg for 5 min at room temperature. The cell pellet was resuspended in breaking buffer (50 mM TrisHCl pH 7.4, 150 mM NaCl) with addition of PMSF (1mM). Cells were disintegrated by vigorous shaking in the presence of glass beads for 10 min at 4°C. After disruption, cell debris were removed by centrifugation at 3,000xg for 5 min. The supernatant was further used for protein determination and lipid extraction.

Lipid analysis- Lipids from total cells were extracted as described by Folch et al. (33) using chloroform/methanol (2:1; v/v) as solvent. For quantification of diacylglycerols (DG), a lipid extract of total cell extracts (200 µg protein) was separated by thin layer chromatography (TLC) using Silica Gel 60 plates (Merck). Chromatograms were developed in an ascending manner using chloroform/acetone/acetic acid (45:4:0.5; per vol.) as a solvent system. Bands were visualized by dipping the plate for 10 s into a solution consisting of 0.63 g $MnCl_2.4H_2O$, 60 mL water, 60 mL methanol, and 4 mL concentrated sulphuric acid and incubated in a heating chamber at 105°C for at least 30 min. Then, DG bands were quantified by densitometric scanning at 400 nm with a TLC Scanner (CAMAG TLC Scanner 3). Diolein served as a standard.

For quantification of total phospholipids, a lipid extract of total cell extracts (800 µg protein) was analyzed by the method of Broekhuyse (34). Individual phospholipids were analyzed from total cell lipid extracts (2 mg protein) by 2-dimensional TLC using chloroform/methanol/25% ammonia (65:35:5; per vol.) as solvent system for the first dimension, and chloroform/acetone/methanol/ acetic acid/water (50:20:10:10:5; per vol.) for

the second dimension. Spots were visualized by staining with iodine vapor, scraped off and quantified by the method of Broekhuyse (34).

Enzyme assays- TG lipase activity of isolated subcellular fractions was determined using LD (5-10 µg protein) or the 30,000xg ER fraction (300-400 µg protein) as an enzyme source. Lipase activity was measured in a final volume of 200 µL. Samples were incubated in a mixture containing 100 mM potassium phosphate buffer, pH 7.5, containing 250 µM [9,10-^{3}H]triolein (specific activity of 33 µCi/ml), 45 µM phosphatidylcholine/phosphatidylinositol (3:1; mol/mol), 25 mM $MgCl_2$ and 0.2% fatty acid free BSA at 30°C for 1 h in a water bath. The substrate was prepared as follows: Triolein and phosphatidylcholine/phosphatidylinositol were dried under a stream of nitrogen and emulsified by sonication for 4 min at 30°C in potassium phosphate buffer with addition of BSA.
The reaction was stopped by adding 3 mL of chloroform/methanol (2:1, v/v), and lipids were extracted by vortexing. The lipid extract was dried under a stream of nitrogen and then dissolved in 40 µl chloroform/methanol (2:1, v/v and separated by TLC. Chromatograms were developed in an ascending manner using chloroform/acetone/acetic acid (45:4:0.5; per vol.) as a solvent system. Fatty acid bands were scraped off the plate and radioactivity was measured by liquid scintillation counting using LSC Safety (Baker, Deventer, The Netherlands) with 5% water as scintillation cocktail.
Lysophospholipid acyltransferase activity of isolated subcellular fractions was measured using 20 µg protein of 30,000xg ER as an enzyme source in a final volume of 200 µL. The assay mixture contained 100 mM TrisHCl, pH 7.5, 1 mM EDTA, 150 mM NaCl, 100 µM lysophosphatidylethanolamine from egg yolk (Sigma) and 20 µM [^{14}C]oleoyl-CoA (41.9 mCi/mmol). Samples were incubated at 30°C for 2 min in a water bath. The reaction was stopped as described above. Chromatograms were developed in an ascending manner using chloroform/acetone/methanol/ acetic acid/water (50:20:10:10:5; per vol.) as a solvent system. Phosphatidylethanolamine bands were scraped off the plate and radioactivity was measured as described above.

RNA isolation and Real Time PCR- Total RNA from cells grown to the mid-logarithmic phase on YPD at 30°C were isolated using RNeasy kit from Qiagen by following the manufacturer's instructions. After DNaseI digestion, Real Time PCR was performed using SuperScript III Platinum SYBR Green One-Step qRT-PCR Kit (Invitrogen) as described by

the manufacturer. Reactions were performed in sealed MicroAmp Optical 96-Well Reaction Plates and amplification was measured using an ABI 7500 instrument (Applied Biosystems). Samples were quantified using the ΔΔCt method described by Livak and Schmittgen (35). With this method differences in mRNA expression after *ACT1* normalization relative to the control can be calculated. Primers used for Real Time-PCR are listed in Table 3.

Fluorescence Microscopy- S. cerevisiae cells were grown in synthetic minimal medium containing glucose to the late logarithmic phase. For the induction of hybrid protein expression, an aliquot of the culture was shifted to galactose containing medium for 4-6 hours. LD were stained with the hydrophobic dye Nile Red (Sigma) (1µg/ml) for 5-10 min at room temperature. Cells were washed twice with sterile water and further used for fluorescence microscopy. Fluorescence microscopy was carried out on a Zeiss Axioskop microscope using a 100× oil immersion objective with a narrow band eGFP and dsRed filter set (Zeiss). Images were taken with a Visicam CCD-camera and displayed using the Metamorph Imaging software (Visitron Systems, Puchheim, Germany). Exposure time for visualization was 10 s for ER proteins and 300 ms for LD proteins. Transmission images were obtained by using Nomarski optics (differential interference contrast, DIC).

Results

Lack of non-polar lipids affects gene expression, protein level and stability of Tgl3p- The major TG lipase Tgl3p from *S. cerevisiae* is a component of LD where it plays a critical role in TG mobilization. Although molecular functions of the enzyme were studied in some detail by Rajakumari et al. (25), evidence about mechanisms regulating the activity of Tgl3p is limited.

A major question addressed in the present study was how Tgl3p behaves in the absence of non-polar lipids and consequently in the absence of LD. We first examined the expression level of *TGL3* under these conditions. As can be seen from Figure 1A, gene expression of *TGL3* was only slightly reduced in a *dga1*Δ*lro1*Δ*are1*Δ*are2*Δ quadruple mutant (QM) which is devoid of LD. This finding was surprising, because the lipase substrate TG is missing in this strain. Interestingly, however, the protein level of Tgl3p in this mutant was markedly lower than in wild type (Figure 1B). Quantitative Western blot analysis revealed a reduction of the total amount of Tgl3p to 50% of wild type (Figure 1C). The strongly reduced amount

of Tgl3p despite the largely unaffected transcription of *TGL3* tempted us to speculate that decreased protein stability might be the reason for the reduced steady state level of the protein. To test this hypothesis, wild type and QM were grown to the mid-logarithmic phase and poisoned with cycloheximide. Then, degradation of Tgl3p in the absence of protein synthesis was monitored over 24 h. Western blot analysis clearly revealed that the stability of Tgl3p was strongly reduced in the absence of LD (Figure 1D). The protein half-life of Tgl3p in the QM was 3 h, whereas that of wild type Tgl3p was approximately 24 h (Figure 1E).

Localization and lipolytic activity of Tgl3p in the absence of LD- In wild type cells, the majority of Tgl3p is located to the surface phospholipid monolayer of LD (Figure 2A). The presence of substantial amounts of Tgl3p in the QM raised the question about the subcellular distribution of the protein in this strain. Previously, it was reported that some LD proteins were retained in the ER of yeast cells lacking non-polar lipids and hence LD (19) reflecting the close relationship of the two organelles. Therefore, we speculated that such a subcellular rearrangement might also occur with Tgl3p. To test this hypothesis, we isolated microsomes from QM and the corresponding wild type strain and tested for the presence of Tgl3p. In wild type, the Myc-tagged Tgl3p was localized to LD at high abundance, confirming that the tag did not influence the subcellular distribution of the protein (Figure 2A). Also other tags did not influence localization and functionality of the enzyme (our own unpublished results). However, smaller amounts of Tgl3-Myc were also detected in 30,000xg (M30) and 40,000xg microsomes (M40) from wild type cells. This result is in contrast to previous studies (20) where a less sensitive assay system did not detect Tgl3p in microsomes. Thus, results presented here demonstrate that Tgl3p also belongs to the group of proteins which are dually located to LD and the ER. The minor signal of Tgl3-Myc in the cytosolic fraction of wild type is most likely due to residual LD. Most importantly, the amounts of Tgl3p present in the homogenate and in microsomes from the QM showed almost the same enrichment pattern as the ER-marker Wbp1p. Thus, in cells lacking LD Tgl3p seems to behave like a “true” ER protein.

Fluorescence microscopy confirmed results obtained by Western blot analysis. For this purpose, we used GFP fused to the N-terminus of Tgl3p under a galactose-inducible promoter. In wild type background, GFP-Tgl3p was located to LD (Figure 2B) and co-localized with Nile Red confirming previous results from our laboratory (20). In the QM,

GFP-Tgl3p was enriched in the nuclear ER exhibiting co-localization with the ER-marker protein Sec61-mCherry.

Lipase activity of Tgl3p in the QM does not seem to be relevant *in vivo,* because the substrate TG is not available. Nevertheless, we tested TG lipolytic activity of Tgl3p when located to the ER. Figure 2C shows *in vitro* TG activity of LD and ER fractions from wild type and QM overexpressing *TGL3*. LD from wild type exhibited a specific lipolytic activity of 0.19 ± 0.045 pmol fatty acids formed/h/mg protein with [9,10-^{3}H]triolein as substrate. However, ER fractions from both wild type and QM showed only marginal TG activity. Thus, it appears that Tgl3p is not an active lipase when located to the ER.

Substrate availability affects regulation of Tgl3p- Experiments described above clearly demonstrated that protein level, stability and localization of Tgl3p are dramatically altered in yeast cells lacking non-polar lipids and LD. The question remained whether or not the absence of the major substrate of Tgl3p, TG, is already sufficient to cause the observed effects. Therefore, we examined gene expression, protein level, stability and localization of Tgl3p in mutants lacking either TG or SE. Czabany et al. (36) had shown that the four non-polar lipid synthesizing enzymes Dga1p, Lro1p, Are1p and Are2p contribute differently to LD formation. The important finding of these authors was that *S. cerevisiae* mutant strains lacking either TG or SE, respectively, are still able to form LD which differed, however, in number, structure, lipid and protein composition. In an *lro1*Δ*are1*Δ*are2*Δ strain where Dga1p is the only active acyltransferase, LD consist entirely of TG, whereas in a *dga1*Δ*lro1*Δ strain both SE synthases are actively producing SE as the only storage lipid of LD. In these strains, gene expression of *TGL3* is only slightly altered compared to wild type (data not shown). However, Western blot analysis of Tgl3p revealed that the amount of the enzyme was reduced in *dga1*Δ*lro1*Δ but not in *lro1*Δ*are1*Δ*are2*Δ (Figure 3A). Quantification of Western blots revealed a reduction of total Tgl3p in *dga1*Δ*lro1*Δ to 60% of wild type and *lro1*Δ*are1*Δ*are2*Δ (Figure 3B). Interestingly, the relative amount of Tgl3p on LD was not changed in all mutants (Figure 3A). The reduced protein level in the *dga1*Δ*lro1*Δ strain, suggested a reduced stability of Tgl3p in cells lacking TG. Indeed, Figures 3C and D show that degradation of Tgl3p in *dga1*Δ*lro1*Δ strain is similar to the QM. In contrast, Tgl3p is rather stable in an *lro1*Δ*are1*Δ*are2*Δ strain. The half-life of Tgl3p was 6 h in *dga1*Δ*lro1*Δ and more than 9 h in *lro1*Δ*are1*Δ*are2*Δ (Figure 3D).

Since experiments described above demonstrated a correlation of protein stability and localization of Tgl3p to the ER, we assumed that the subcellular distribution of Tgl3p was also affected in a yeast strain lacking TG. Indeed, a large portion of the lipase was found in the ER of a *dga1*Δ*lro1*Δ strain which contains SE as the only non-polar lipid in LD (Figure 3E; M30 and M40 fractions), although Tgl3p was still present in LD. In the *dga1*Δ*lro1*Δ strain, the enrichment of Tgl3p in microsomal fractions over the homogenate (Hom) was similar to the ER marker protein Wbp1p. However, it has to be noted that the amount of LD in *dga1*Δ*lro1*Δ is markedly lower than in wild type yeast cells (our own unpublished observations; and ref. (36)). Thus, the low number of LD in this mutant and its altered non-polar lipid composition may cause the altered subcellular localization of Tgl3p in *dga1*Δ*lro1*Δ. In sharp contrast, Tgl3p was not enriched in microsomes from the *lro1*Δ*are1*Δ*are2*Δ strain which contains LD with TG as the only non-polar lipid. In this mutant, the subcellular distribution of Tgl3p was the same as in wild type.

Gene expression, stability and localization of Tgl3p variants bearing mutations in the two active centers of the enzyme- As described above considerable amounts of Tgl3p are present in yeast cells lacking TG and even the storage compartment for non-polar lipids, the LD. This finding raised the question as to the function of Tgl3p in the absence of its substrate. Rajakumari et al. (25) demonstrated that Tgl3p not only acts as a lipase but also as an acyltransferase mediating acyl-CoA dependent acylation of lysophospholipids. These two enzymatic activities are catalyzed by two independent active centers. Therefore, we speculated that lack of TG and shift of Tgl3p from LD to the ER might favor the second function of the protein, the acyltransferase activity. To address this question, we tested Tgl3p variants bearing mutations in one of the active centers for gene expression, protein level and localization. A lipase defective mutant $Tgl3^{S237A}$, an acyltransferase defective $Tgl3^{H298A}$ and a $Tgl3^{S237A;H298A}$ mutant bearing mutations in both active centers were analyzed. qRT-PCR analysis revealed no significant changes in mRNA levels of yeast strains expressing the different variants of *TGL3* compared to wild type (Figure 4A). Furthermore, protein levels of mutated Tgl3p proteins were not changed (Figure 4B). Finally, none of the tested mutations at the active centers of Tgl3p affected localization of the enzyme to LD, since signals of HA-Tgl3p variants were still highly enriched in LD fractions (Figure 4C). Concomitantly, the occurrence of Tgl3p variants in microsomal fractions was not affected (Figure 4D). Thus, we

conclude that changes in the enzymatic activities of Tgl3p introduced by the above mentioned mutations did not influence formation and localization of the enzyme.

TG substrate availability affects contribution of Tgl3p to phospholipid synthesis- Since Tgl3p appears to play also a role in phospholipid metabolism of *S. cerevisiae* (25), we tested its possible contribution to phospholipid synthesis in the absence of non-polar lipids. To address this question we performed phospholipid analysis of different mutants. Figure 5A shows the amount of total phospholipids in wild type, QM, *lro1*Δ*are1*Δ*are2*Δ and *dga1*Δ*lro1*Δ strains with an additional deletion of *TGL3*, respectively. These results confirmed the role of Tgl3p in phospholipid synthesis in wild type background, since the amount of phospholipids was reduced to 80% of wild type in a *tgl3*Δ mutant. Interestingly, however, deletion of *TGL3* in the QM background did not change the amount of total phospholipids. We also tested *in vitro* acyltransferase activity of Tgl3p in a 30,000xg ER fraction from QM and QM*tgl3*Δ. Figure 5B shows that deletion of *TGL3* in the QM background did not change lysophosphatidylethanolamine acyltransferase activity. Similar measurements with wild type and *tgl3Δ* did not show any differences either (data not shown). These results indicate that Tgl3p does not play a significant role in phospholipid synthesis when located to the ER. Apparently, Tgl3p requires the presence of TG to contribute efficiently to phospholipid synthesis. To address this question in more detail we determined the amount of total phospholipids in *lro1*Δ*are1*Δ*are2*Δ and *dga1*Δ*lro1*Δ strains with and without additional deletion of *TGL3*. Deletion of *TGL3* resulted in a slightly reduced phospholipid level in the *lro1*Δ*are1*Δ*are2*Δ background when LD contained TG, but not in a *dga1*Δ*lro1*Δ strain where SE are the only non-polar lipid components of LD. To further investigate a possible role of *TGL3* in phospholipid synthesis, we analyzed the phospholipid composition of wild type, QM, *lro1*Δ*are1*Δ*are2*Δ and *dga1*Δ*lro1*Δ strains with an additional deletion of *TGL3*, respectively (Table 4). These analyses revealed that the phospholipid composition of wild type and *lro1*Δ*are1*Δ*are2*Δ were clearly different from *dga1*Δ*lro1*Δ and QM strain. In *dga1*Δ*lro1*Δ and the QM the amount of phosphatidylinositol and phosphatidylserine is markedly reduced compared to wild type and *lro1*Δ*are1*Δ*are2*Δ. In contrast, phosphatidylethanolamine and phosphatidylcholine were increased in *dga1*Δ*lro1*Δ and QM strain, respectively. A slight increase of lysophospholipids was measured when *TGL3* was deleted in wild type and *lro1*Δ*are1*Δ*are2*Δ background. In contrast, no enrichment of lysophospholipids was observed in *dga1*Δ*lro1*Δ and the QM. These data indicate that Tgl3p

requires LD with both TG and SE or at least the presence of TG to serve as a lysophospholipid acyltransferase.

Kurat et al. (22) demonstrated, that Tgl3p preferentially hydrolyzes TG but also exhibits minor DG lipolytic activity. Therefore, we speculated that Tgl3p may act as DG lipase in the absence of TG. Figure 5C shows the amounts of DG in strains lacking *TGL3* in wild type and QM background. Compared to wild type and *tgl3*Δ, QM and QM*tgl3*Δ strains show higher levels of DG. The increased amount of DG in these mutants can be explained by the lack of TG synthases, rendering strains unable to convert DG to TG. Additionally, the fatty acid composition of DG is slightly altered in the QM compared to wild type (data not shown). However, deletion of *TGL3* in the QM background did not further affect the DG level (Figure 5C) and the fatty acid composition of DG (data not shown). Thus, Tgl3p does not gain relevant DG hydrolytic activity *in vivo* when TG is missing. Altogether, Tgl3p appears to be rather inactive in the absence of LD and after a shift to the ER. Thus, the ER may be regarded as a parking lot for the yeast TG lipase Tgl3p.

Discussion

The major TG lipase Tgl3p from *S. cerevisiae* LD plays a critical role in TG mobilization, but also contributes to phospholipid metabolism (20, 23). Although the biochemistry of this enzyme has been studied in some detail (25), little is known about regulation of Tgl3p activity. Here, we provide some insight into regulatory aspects of the TG metabolic network in *S. cerevisiae* with emphasis on the role of Tgl3p. As possible mechanisms regulating the activity of Tgl3p, transcriptional and translational control, protein stability and subcellular localization of the enzyme were anticipated. As another very important regulatory aspect the substrate availability was considered as well as a possible feedback control by the products formed. Finally, posttranslational modifications or direct inhibitory or stimulating effects on the enzyme level may play a role for the activity of Tgl3p.

In this study we show that regulation of Tgl3p activity on the gene expression level is of minor importance. Our data demonstrated only minor changes of *TGL3* expression in wild type and mutants lacking non-polar lipids and consequently LD (see Figure 1A) or bearing mutations in the active centers of the enzyme (see Figure 4A).

A more important regulatory mechanism is the protein stability of the TG lipase Tgl3p especially in the absence of LD (see Figure 1). Sorger et al. (19) had demonstrated that

squalene epoxidase Erg1p, another protein which is dually located to LD and the ER, is stable only when located to LD. In the absence of LD, i.e., in a *dga1*Δ*lro1*Δ*are1*Δ*are2*Δ mutant, the stability of Erg1p was strongly compromised. A stabilizing effect of LD on the polypeptide was suggested. Thus, stabilization of proteins by association with the LD surface membrane may not only be an exclusive effect for one or two proteins such as Tgl3p and Erg1p, but probably a more general phenomenon. With this regard, the topology of LD proteins and their assembly into the surface monolayer of LD may play an important role. Additionally, the absence or presence of TG, the major substrate of Tgl3p, seems to cause marked changes in protein stability (see Figure 3).

Previous studies had already shown that some typical LD proteins are located to the ER in the absence of LD (10, 19). These observations supported the view that LD and ER are closely related subcellular fractions. Thus, it was not surprising that also Tgl3p was localized to the ER in a QM. The dual localization of Tgl3p to LD and the ER in wild type (see Figure 2A) has not been explicitly shown before but is in line with other reports about LD proteins (37). More surprising was the finding, that absence or presence of the major lipase substrate TG affected the distribution of Tgl3p between LD and ER (see Figure 3E). LD from yeast strains lacking one or more non-polar lipid synthesizing enzymes vary in lipid composition, structure, size and number (36). Moreover, it was reported that the lipid composition of LD affects the protein equipment of the organelle. Thus, the absence of TG appears to have such an effect on Tgl3p. It has to be noted, however, that TG are not essential for Tgl3p localization to LD, but in the absence of TG the number and size of LD is significantly reduced which causes a sort of overflow of the enzyme to the ER.

As described above, lack of LD or depletion of TG causes complete or partial re-localization of Tgl3p to the ER. This re-localization may be explained by the functional and biosynthetic link of LD and the ER. It appears that Tgl3p is retained to the ER when LD are missing or insufficiently equipped with TG. Entire or partial re-localization of Tgl3p to the ER always results in a loss of protein stability. This effect may be due to the inappropriate embedding and altered topology of Tgl3p in the ER bilayer membrane. Our preliminary results (unpublished data) indicate that Tgl3p located to the ER becomes more accessible to proteolytic digestions than in the monolayer membrane of LD.

Tgl3p was shown to act not only as TG lipase but also as an lysophospholipid acyltransferase (25). The two activities of the enzyme are independent of each other and catalyzed by two distinct active centers. This finding led us to speculate that these two enzymatic activities

might also be regulated independently. Most surprisingly our results demonstrated that Tgl3p needs to be located to LD with TG present to contribute to phospholipid synthesis *in vivo* (see Figure 5A and Table 4). As presence of the lipase substrate TG seems to be important also for the acyltransferase activity, we speculate that TG degradation and lysophospholipid acylation catalyzed by Tgl3p are linked processes. *In vitro* Tgl3p utilizes acyl-CoAs for efficient acylation of lysophosphatidylethanolamine, indicating that fatty acyl activation is required for this phospholipid biosynthetic route. Indeed, such fatty acid activating enzymes are present on the surface of LD which might contribute to this second activity of Tgl3p (5, 6). Additionally, the two other TG lipases of the yeast, Tgl4p and Tgl5p, might also play a role in providing fatty acids for phospholipid biosynthesis on LD. Similar to Tgl3p, these two lipases are lysophospholipid acyltransferases (24, 25). Furthermore, interaction between LD and the ER may be required for a concerted action in phospholipid biosynthesis (10).

In summary, our results demonstrate that TG substrate limitation in the yeast causes changes in Tgl3p stability, gene expression and localization. Moreover, a link between TG lipolysis and the capacity of Tgl3p to perform acyltransferase reactions was shown. These findings are novel facets in the regulatory network of non-polar lipid metabolism.

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Abbreviations: DG, diacylglycerols; ER, endoplasmic reticulum; GFP, green fluorescent protein; LD, lipid droplets; QM, quadruple mutant; SE, steryl esters; TG, triacylglycerols; TLC, thin layer chromatography.

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Tables

Table1 Yeast strains used in this study

Strain	Genotype	Source
Wild type	BY4741 *Mat a; his3Δ1; leu2Δ0; met15Δ0; ura3Δ0*	Euroscarf
QM	BY4741; *dga1*Δ::*kanMX4 lro1*Δ::*kanMX4 are1*Δ::*kanMX4 are2*Δ::*kanMX4*	(38)
*tgl3*Δ	BY4741; *tgl3*Δ::*kanMX4*	Euroscarf
QM*tgl3*Δ	QM; *tgl3*Δ::*HIS3MX6*	This study
*lro1*Δ*are1*Δ*are2*Δ	BY4741; *lro1*Δ::*kanMX4 are1*Δ::*kanMX4 are2*Δ::*kanMX4*	K. Athenstaedt
*dga1*Δ*lro1*Δ	BY4741; *lro1*Δ::*kanMX4 dga1*Δ::*HIS3MX6*	(39)
*lro1*Δ*are1*Δ*are2*Δ*tgl3*Δ	BY4741; *lro1*Δ::*kanMX4 are1*Δ::*kanMX4 are2*Δ::*kanMX4 tgl3*Δ::*URA3KL*	This study
*dga1*Δ*lro1*Δ*tgl3*Δ	BY4741; *lro1*Δ::*kanMX4 dga1*Δ::*HIS3MX6 tgl3*Δ::*URA3KL*	This study
Tgl3-Myc	BY4741; *TGL3-13Myc*::*HIS3MX6*	This study
QM Tgl3-Myc	QM; *TGL3-13Myc*::*HIS3MX6*	This study
GFP-Tgl3	BY4741; *HIS3MX6*::*PGal1-GFP*(*S65T*)-*TGL3*	(20)
QM GFP-Tgl3	QM; *HIS3MX6*::*PGal1-GFP*(*S65T*)-*TGL3*	This study
*lro1*Δ*are1*Δ*are2*Δ Tgl3-Myc	BY4741; *lro1*Δ::*kanMX4 are1*Δ::*kanMX4 are2*Δ::*kanMX4 TGL3-13Myc*::*HIS3MX6*	This study
*dga1*Δ*lro1*Δ Tgl3-Myc	BY4741; *lro1*Δ::*URA3KL dga1*Δ::*kanMX4 TGL3-13Myc*::*HIS3MX6*	This study

Table 2 Primers used throughout this study

Primer	Sequence (5'→3')
Tgl3F1fwd	GTGCAGTCGAATTTAAATTAGACGACATAATAAGAG CAAGACGGAGTAGGCGGATCCCCGGGTTAATTAA
Tgl3S2rev	ATCGAGCTCTATCAATAAAAAAAATAAGACAGAAAA AAGTGGAAACGATAATCGATGAATTCGAGCTCG
Tgl3S1fwd	AATCATCTATTCATATATCACATCTTTGAGTTGCCGTT AAGCATGCGTACGCTGCAGGTCGAC
Tgl3-GFP (N-term.) fwd	ATGACACAATAGAAAGGGAATCATCTATTCATATATC ACATCTTTGAGTTGCCGTTAAGCGAATTCGAGCTCGT TTAAAC
Tgl3-GFP (N-term.) rev	CCAGTTTTTCAAAAGGGTCGGTATTACAGCAGACACC TTGTATTCCTGCGCCGTTTCCTTTTGTATAGTTCATCC ATGC
Lro1S1fwd	AGCCATTACAAAAGGTTCTCTACCAACGAATTCGGCG ACAATCGAGTAAAAAATGCGTACGCTGCAGGTCGAC
Lro1S2rev	TCTTTTCGCTCTTTGAAATAATACACGGATGGATAGT GAGTCAATGTCGGTCATTTAATCGATGAATTCGAGCT CG
*BamH*ISec61Fwd	TATAGGATCCATGTCCTCCAACCGTGTTC
Sec61*Not*IcherryfusRev	CTCGCCCTTGCTCACCATGCGGCCGCACATCAAATCA GAAAATCCTGGA
Sec61*Not*IcherryfusFwd	TCCAGGATTTTCTGATTTGATGTGCGGCCGCATGGTG AGCAAGGGCGAG
Cherry*Xho*IRev	TATACTCGAGTTACTTGTACAGCTCGTCCAT
Tgl3fwd*BamH*I	AAAAGGATCCATGTACCCATACGATGTTCCTGACTAT GCGAAGGAAACGGCGCAGG
Tgl3rev*Pst*I	AAAACTGCAGCTACAAGTCTTCCTCGGAGATTAGCTT TTGTTCCCTACTCCGTCTTGCTCTTA

Table 3 Primers used for RT-PCR

Primer	Sequence (5'→3')
RT Act1-fwd	CCAGCCTTCTACGTTTCCATCCAAG
RT Act1-rev	GACGTGAGTAACACCATCACCGGA
RT Tgl3-fwd	GCCAACAATCCGAGCATAACGGAG
RT Tgl3-rev	TGGTGCCAAGTATGGTCTCGCCA

Table 4 Phospholipid composition of cell free homogenate from cells grown on YPD. WT, wild type; QM, quadruple mutant; LPL, lysophospholipids; PI, phosphatidylinositol, PS, phosphatidylserine; PE phosphatidylethanolamine; PC, phosphatidylcholine; DMPE, dimethylphosphatidylethanolamine; CL, cardiolipin; PA, phosphatidic acid. Mean values of at least three independent samples with standard deviations are shown.

Strain	Phospholipids in cell free homogenate (mol %)							
	LPL	PI	PS	PC	PE	DMPE	CL	PA
WT	1.0 ± 0.7	16.6 ± 2.3	6.3 ± 1.1	47.6 ± 2.1	21.3 ± 1.4	2.6 ± 0.4	1.6 ± 0.4	3.2 ± 0.6
*tgl3*Δ	1.8 ± 0.9	16.6 ± 1.5	7.3 ± 0.5	44.1 ± 1.2	22.0 ± 0.8	3.4 ± 0.4	1.7 ± 0.2	2.7 ± 0.8
*lro1*Δ*are1*Δ*are2*Δ	0.9 ± 0.6	19.1 ± 4.1	7.5 ± 0.7	46.9 ± 4.3	18.7 ± 0.9	2.6 ± 0.9	1.5 ±0.2	2.8 ± 0.5
*lro1*Δ*are1*Δ*are2*Δ*tgl3*Δ	2.4 ± 0.1	16.8 ± 2.2	7.1 ± 1.3	46.5 ± 2.2	18.6 ± 1.1	3.5 ± 0.3	2.1 ± 0.1	3.0 ± 0.2
*dga1*Δ*lro1*Δ	1.7 ± 1.0	7.8 ± 1.6	4.4 ± 0.3	51.5 ± 3.0	26.6 ± 0.7	3.8 ± 0.6	1.2 ± 0.2	3.1 ± 0.3
*dga1*Δ*lro1*Δ *tgl3*Δ	1.2 ± 0.5	6.2 ± 2.1	4.6 ± 2.0	51.0 ± 2.4	26.7 ± 2.3	5.5 ± 0.8	1.2 ± 0.2	3.8 ± 0.4
QM	0.8 ± 0.6	8.0 ± 1.3	2.5 ± 1.0	56.6 ± 2.4	23.5 ± 1.0	6.1 ± 0.7	1.1 ± 0.3	1.5 ± 0.2
QM*tgl3*Δ	1 ± 0.9	6.7 ± 1.7	2.1 ± 0.2	58.7 ± 2.7	23.2 ± 0.9	6.0 ± 0.9	1.1 ± 0.6	1.3 ± 0.7

FIGURE 1

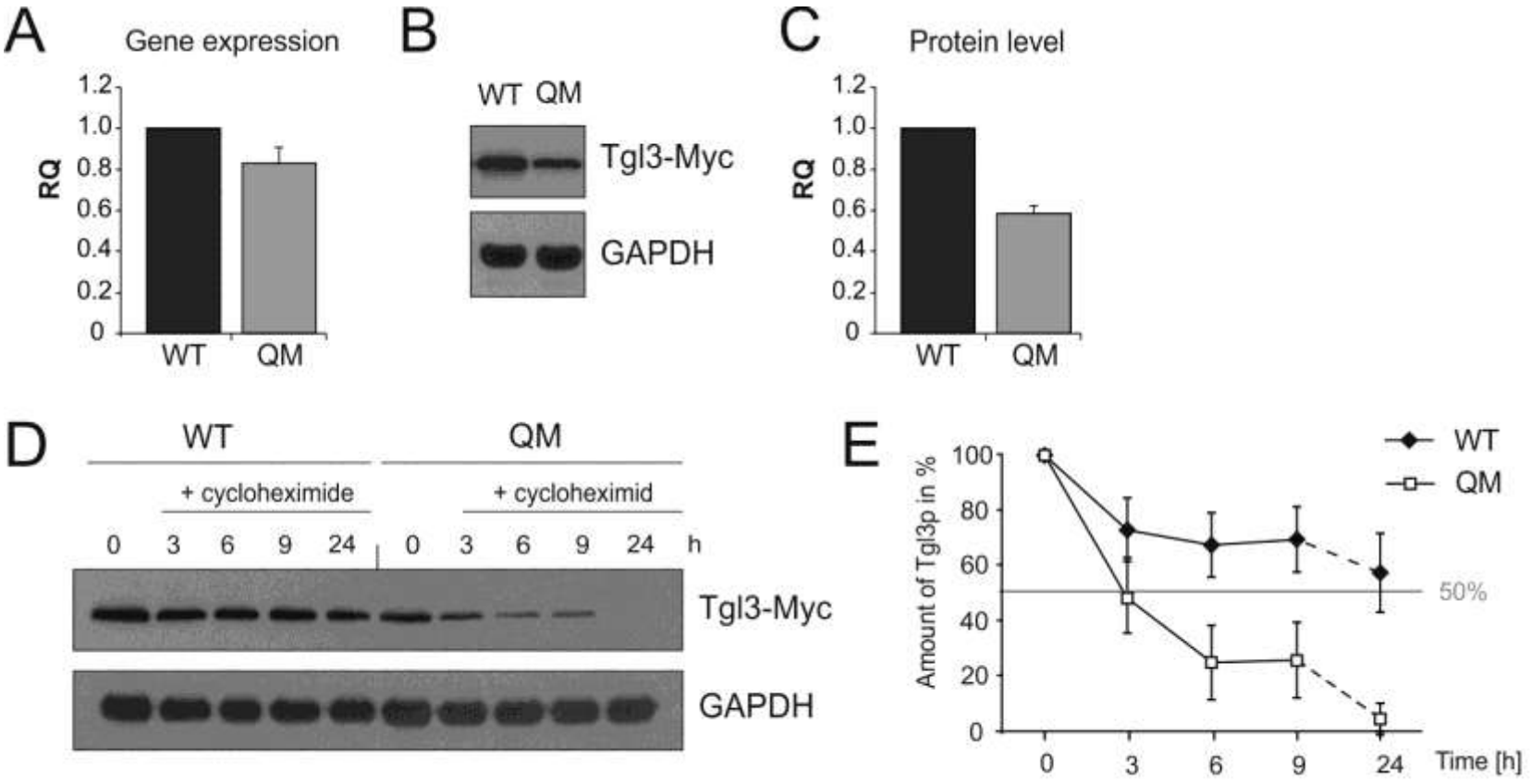
A
Gene expression
RQ
1.2
1.0
0.8
0.6
0.4
0.2
0
WT
QM
B
WT QM
Tgl3-Myc
GAPDH
C
Protein level
RQ
1.2
1.0
0.8
0.6
0.4
0.2
0
WT
QM
D
WT
QM
+ cycloheximide
+ cycloheximid
0 3 6 9 24 0 3 6 9 24 h
Tgl3-Myc
GAPDH
E
WT
QM
Amount of Tgl3p in %
100
80
60
40
20
0
50%
0 3 6 9 24
Time [h]

Fig. 1 Gene expression, protein level and stability of Tgl3p in the absence of LD. A. Relative gene expression of *TGL3* in wild type (WT) (black bar) and QM (grey bar) measured by RT-PCR. Wild type was set at 1. Data are mean values from three independent experiments with the respective deviation. B. Protein analysis of Tgl3-Myc of total cell extracts from wild type and QM grown to the stationary phase. C. The relative protein level of Tgl3-Myc of total cell extracts from wild type (black bar) and QM (grey bar) obtained by three Western blots was calculated using ImageJ program. D. Western blot analysis of Tgl3-Myc was performed with total cell extracts from wild type and QM grown for time periods as indicated after addition of 100 µg/mL cycloheximide to cells grown to mid-logarithmic phase. GAPDH was used as loading control. E. The relative protein stability in wild type and QM obtained by three Western blots was calculated using ImageJ program. Protein half-life is shown. Western blot analyses are representative of at least two independent experiments. RQ, relative quantity.

FIGURE 2

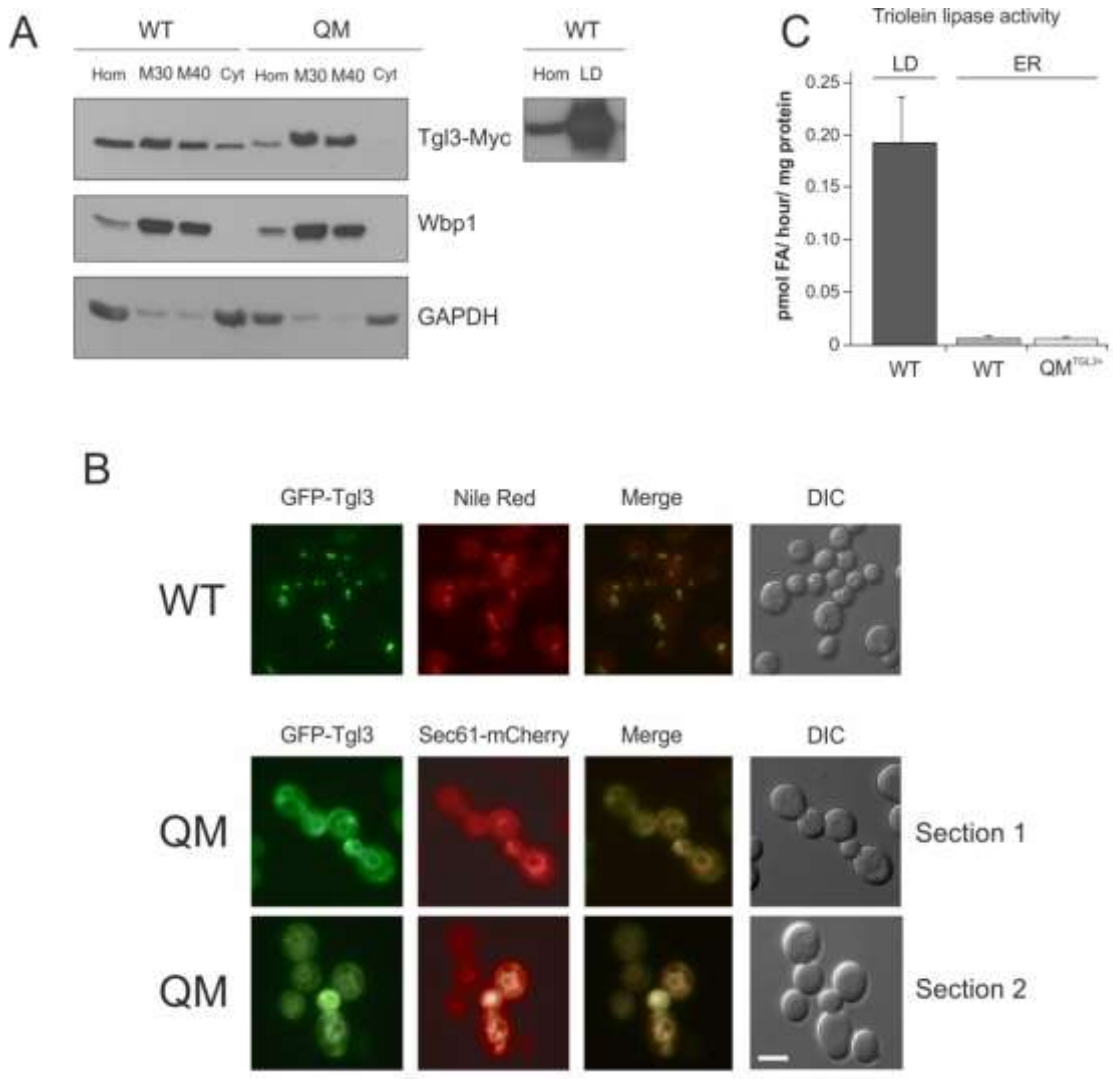
A
WT
QM
WT
Hom M30 M40 Cyt Hom M30 M40 Cyt
Hom LD
Tgl3-Myc
Wbp1
GAPDH
C
Triolein lipase activity
LD
ER
pmol FA/ hour/ mg protein
0.25
0.20
0.15
0.10
0.05
0
WT
WT
QM
B
GFP-Tgl3
Nile Red
Merge
DIC
WT
GFP-Tgl3
Sec61-mCherry
Merge
DIC
QM
Section 1
QM
Section 2

Fig. 2 Localization and lipase activity of Tgl3p in the absence of LD. A. Western blot analysis of Tgl3-Myc in homogenate (Hom), 30,000xg microsomes (M30), 40,000xg microsomes (M40), cytosol (Cyt) and LD fraction (LD) from wild type (WT) and QM grown to the stationary phase. Primary antibodies were directed against the Myc-tag, Wbp1p (ER-marker) and GAPDH (cytosolic marker). Western blot analyses are representative of at least two independent experiments. B. Fluorescence microscopy of PGal1-GFP-Tgl3 in wild type and QM grown to late logarithmic phase after induction with galactose for 4 hours. Two different sections from the QM strain are shown. Size bar: 5 µm. C. Analysis of TG lipase activity of LD and 30,000xg ER fractions from wild type and QM overexpressing *TGL3*. Experiments were performed in triplicates and are representative of at least two independent experiments. Data are mean values with the respective deviation.

FIGURE 3

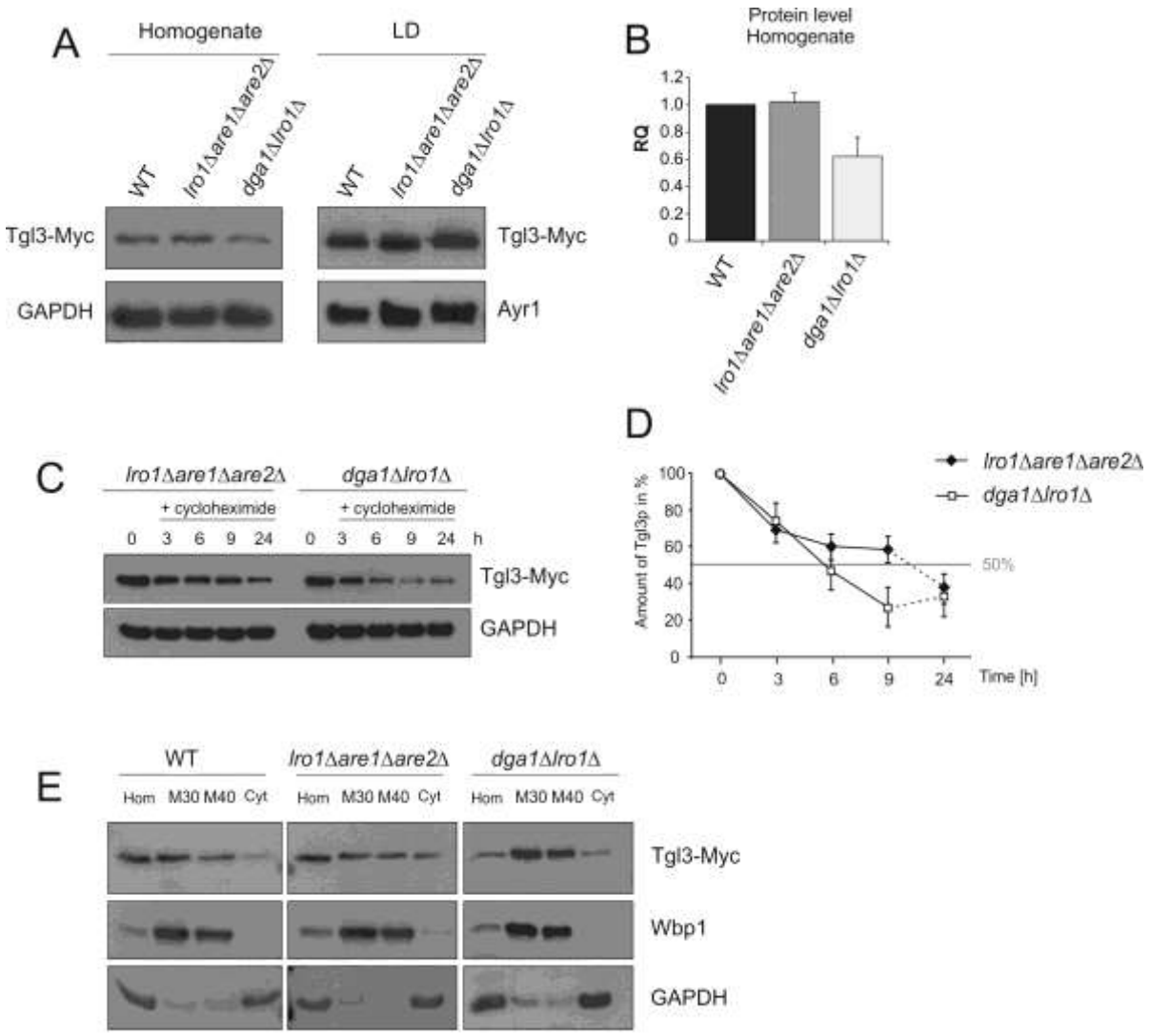
A
Homogenate
LD
WT
lro1Δare1Δare2Δ
dga1Δlro1Δ
Tgl3-Myc
GAPDH
Ayr1
B
Protein level
Homogenate
RQ
C
+ cycloheximide
0 3 6 9 24 h
D
Amount of Tgl3p in %
50%
Time [h]
E
Hom M30 M40 Cyt
Wbp1

Fig. 3 Localization of Tgl3p in yeast strains lacking non-polar lipid synthesizing enzymes. A. Western blot analysis of Tgl3-Myc in homogenate and LD fractions from wild type (WT), *lro1*Δ*are1*Δ*are2*Δ and *dga1*Δ*lro1*Δ grown to the stationary phase. B. The relative protein levels of Tgl3-Myc from total cell extracts of wild type (black bar), *lro1*Δ*are1*Δ*are2*Δ (grey bar) and *dga1*Δ*lro1* (light grey bar) obtained by three Western blots were calculated using ImageJ program. C. Western blot analysis of Tgl3-Myc was performed with total cell extracts from *lro1*Δ*are1*Δ*are*Δ and *dga1*Δ*lro1*Δ grown for time periods as indicated after addition of 100 µg/mL cycloheximide to cells grown to the mid-logarithmic phase. D. The relative protein stability in *lro1*Δ*are1*Δ*are*Δ and *dga1*Δ*lro1*Δ obtained by three Western blots was calculated using ImageJ program. Protein half-life is shown. E. Western blot analysis of Tgl3-Myc in homogenate (Hom), 30,000xg microsomes (M30), 40,000xg microsomes (M40) and cytosol (Cyt). Primary antibodies were directed against the Myc-tag, Wbp1p (ER-marker), GAPDH (cytosolic marker), Ayr1p (LD marker) and Erg1p (LD marker). Western blot analyses are representative of at least two independent experiments. RQ, relative quantity.

FIGURE 4

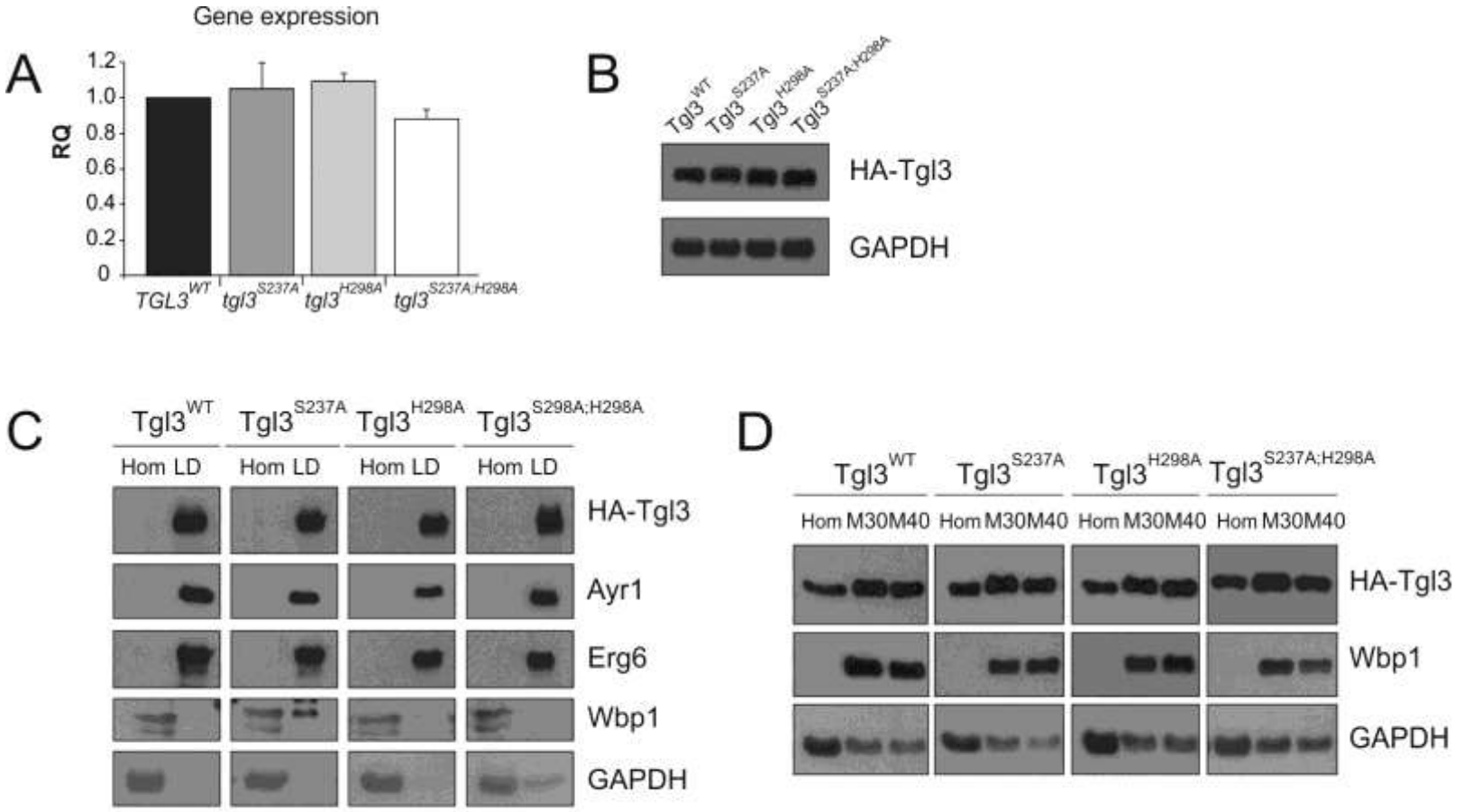
Gene expression
A
RQ
1.2
1.0
0.8
0.6
0.4
0.2
0
TGL3WT
tgl3S237A
tgl3H298A
tgl3S237A;H298A
B
Tgl3WT
Tgl3S237A
Tgl3H298A
Tgl3S237A;H298A
HA-Tgl3
GAPDH
C
Tgl3WT
Tgl3S237A
Tgl3H298A
Tgl3S298A;H298A
Hom LD
HA-Tgl3
Ayr1
Erg6
Wbp1
GAPDH
D
Tgl3WT
Tgl3S237A
Tgl3H298A
Tgl3S237A;H298A
Hom M30M40
HA-Tgl3
Wbp1
GAPDH

Fig. 4 Gene expression, protein level and localization of Tgl3p in different mutants. A. Relative gene expression of *TGL3*$^{wild\ type}$, *tgl3*^{S237A}, *tgl3*^{H298A} and *tgl3*$^{S237A;H298A}$ was measured by RT-PCR. *TGL3*$^{wild\ type}$ was set at 1. Data are mean values from three independent experiments with the respective deviations. B. Protein analysis of Tgl3$^{wild\ type}$, Tgl3^{S237A}, Tgl3^{H298A} and Tgl3$^{S237A;H298A}$ from total cell extracts grown to stationary phase. GAPDH was used as loading control. C. Western blot analysis of Tgl3$^{wild\ type}$, Tgl3^{S237A}, Tgl3^{H298A} and Tgl3$^{S237A;H298A}$ in homogenate (Hom) and LD fractions from cells grown to the stationary phase. D. Western blot analysis of Tgl3p and variants in homogenate (Hom), 30,000xg microsomes (M30) and 40,000xg microsomes (M40). Primary antibodies were directed against the HA-tag, Wbp1p (ER-marker), GAPDH (cytosolic marker), Ayr1p (LD marker) and Erg6p (LD marker). Western blot analyses are representative of at least two independent experiments. RQ, relative quantity.

FIGURE 5

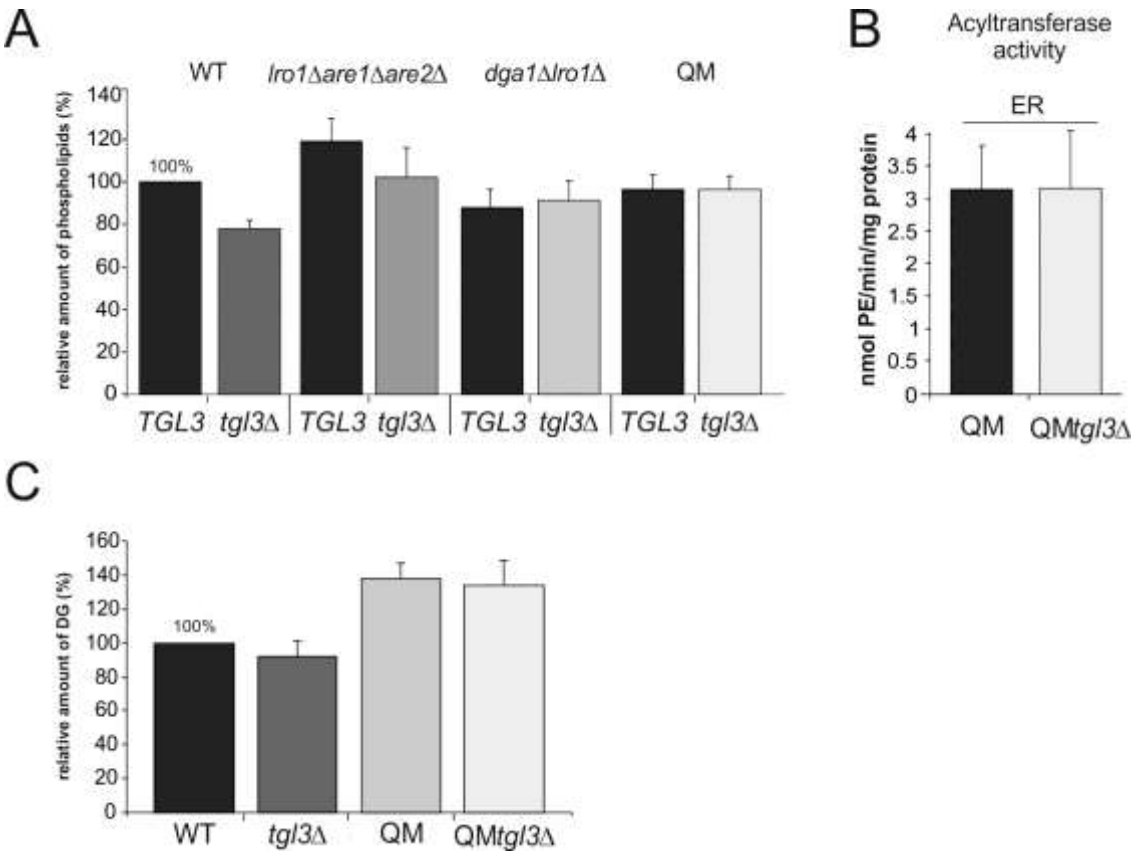

Fig. 5 Lipid analysis of yeast strains lacking major non-polar lipid synthesizing enzymes. A. Relative amounts of total phospholipids/mg protein of total cell extracts from wild type (WT) and mutants with additional deletion of *TGL3* grown to the stationary phase. Data are mean values of three independent experiments with respective deviations. The wild type (45 mg ± 4.9 mg phospholipids/mg protein) was set at 100%. B. Analysis of lysophosphatidylethanolamine acyltransferase activity in 30,000xg ER fractions from QM and QM*tgl3*Δ. Assays were performed in triplicate from at least two independent biological samples. Data are mean values with the respective deviation. C. Relative amount of DG from wild type and QM with additional deletion of *TGL3* in cells grown to the stationary phase. Data are mean values of three independent experiments with respective mean deviations. Wild type was set at 100%.

CHAPTER IV

Defects in triacylglycerol lipolysis affect synthesis of triacylglycerols and steryl esters in the yeast

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Keywords: Triacylglycerol, lipase, steryl ester, lipid droplet, yeast

Abstract

Tgl3p, Tgl4p and Tgl5p are the major triacylglycerol lipases of the yeast *S. cerevisiae* catalyzing degradation of triacylglycerols stored in lipid droplets. Previous results from our laboratory (Athenstaedt and Daum, 2005, *J. Biol. Chem.* 280, 37301–37309) demonstrated that a yeast strain lacking all three triacylglycerol lipases accumulates triacylglycerols at high amount, but also steryl esters. Here we show a metabolic link between synthesis and mobilization of non-polar lipids. In particular, we demonstrate that a block in triacylglycerol degradation in a *tgl3*Δ*tgl4*Δ*tgl5*Δ triple mutant lacking all major triacylglycerol lipases causes marked changes in non-polar lipid synthesis. Under these conditions formation of triacylglycerols is reduced, whereas steryl ester synthesis is enhanced as shown by quantification of non-polar lipids, *in vivo* labeling of lipids using [^{14}C]oleic and [^{14}C]acetic acid as precursors, and enzyme analyses *in vitro*. In summary, this study demonstrates that triacylglycerol and steryl ester metabolism are linked processes. The importance of balanced storage and degradation of these components for lipid homeostasis in the yeast is highlighted.

Introduction

The major non-polar lipids triacylglycerols (TG) and steryl esters (SE) are depot forms of metabolic energy. In the yeast *S. cerevisiae,* however, fatty acids and sterols stored in TG and SE, respectively, are mainly used as building blocks for membrane biosynthesis [1,2]. Furthermore, formation of non-polar lipids "neutralizes" a possible toxic effect of free fatty acids and sterols. TG and SE form the hydrophobic core of a unique organelle called lipid droplet (LD), lipid particle, lipid body, oil body or oleosome [1–3]. In *S. cerevisiae* the core of LD is formed from TG and SE at similar amounts [4]. This non-polar lipid core is enwrapped by a phospholipid monolayer with a small amount and number of proteins embedded [5,6]. During the last decade the protein equipment of LD has been well studied, and LD proteins were characterized in some detail [6,7]. Most of the LD proteins are involved in lipid metabolism, although other cellular functions were also attributed to LD such as storage and degradation of protein aggregates or proteins which are incorrectly folded [8].

Non-polar lipid synthesizing enzymes have been studied in detail not only in *S. cerevisiae* but also in other yeasts [9–12]. The four acyltransferases Dga1p, Lro1p, Are1p and Are2p contribute to TG and SE synthesis in *S. cerevisiae* [13]. Dga1p is the major TG synthase under standard growth condition in the stationary growth phase [9,14]. This acyl-CoA:diacylglycerol acyltransferase requires an activated fatty acid (acyl-CoA) for the esterification of diacylglycerols and shows a dual localization to the ER and LD. The highest specific Dga1p acyltransferase activity *in vitro* was measured in LD [15]. The second TG synthase is encoded by *LRO1* (lecithin:cholesterol acyltransferase related open reading frame) [16,17]. This phospholipid:diacylglycerol acyltransferase is located to the ER and catalyzes an acyl-CoA independent diacylglycerol acyltransferase reaction. Lro1p requires a phospholipid, preferentially phosphatidylethanolamine or phosphatidylcholine, as acyl-donor for TG synthesis. In contrast to Dga1p, Lro1p seems to be the major TG synthase in the logarithmic growth phase of yeast cells [14].

The two acyl-CoA:sterol acyltransferases Are1p and Are2p which are localized to the ER [18,19] are members of the membrane-bound *O*-acyltransferase family [20,21]. Whereas Are2p prefers ergosterol as a substrate, Are1p uses ergosterol and ergosterol precursors, mainly lanosterol as substrates. *In vitro* Are2p was verified as the major SE synthase accounting for 65-75% of total acyl-CoA:sterol acyltransferase activity [22]. Additionally, the two SE synthases show minor acyl-CoA:diacylglycerol acyltransferase activity and thus may contribute to TG synthesis, although with minor efficiency [23].

Tgl3p, Tgl4p and Tgl5p are the major TG lipases of the yeast *S. cerevisiae* and responsible for most of the TG degradation from LD [24–26]. Tgl3p was shown to be the most potent TG lipase with a broad specificity to various TG species with different acyl chains [25]. Deletion of *TGL3* resulted in a markedly increased TG level compared to wild type and an altered fatty acid composition of TG. Myristic acid and palmitic acid were slightly increased in a *tgl3*Δ strain, whereas stearic acid and oleic acid were decreased. Furthermore, the long chain fatty acid hexacosanoic acid was enriched in TG from this mutant. A single deletion of *TGL4* resulted in a moderate increase of total TG compared to wild type whereas a single deletion of *TGL5* did not change the overall TG content at all. Interestingly, Tgl3p, Tgl4p and Tgl5p do not only act as TG lipases but also exhibit lysophospholipid acyltransferase activities as shown by recent studies from our laboratory [27–30].

Little is known about regulation of non-polar lipid metabolism. It was speculated that synthesis and mobilization of these components are balanced because a *tgl3*Δ*tgl4*Δ*tgl5*Δ triple mutant (TM) lacking the major TG lipases did not only accumulate TG at high amount (~2.4-fold increased over wild type), but also SE (~1.4-fold increased over wild type) [25]. These findings led us to investigate the metabolic link between synthesis and degradation of non-polar lipids in the yeast in more detail. In the present study, we demonstrate that TG and SE metabolism are indeed linked processes. These results were obtained by non-polar lipid analysis, *in vivo* labeling of lipids using [^{14}C]oleic and [^{14}C]acetic acid as lipid precursors, and enzyme analyses *in vitro*. The importance of these findings for balanced formation and degradation of non-polar lipids and the overall lipid homeostasis in the yeast will be discussed.

Materials and Methods

Strains and culture conditions

Strains used throughout this study are listed in Table 1. Cells were cultivated aerobically to either the logarithmic or the stationary growth phase in YPD media containing 1% yeast extract, 2% glucose and 2% peptone at 30°C. Yeast strains bearing plasmids were cultivated in synthetic minimal medium containing 0.67% yeast nitrogen base (U.S. Biochemical Corp.), 2% glucose, and the respective amino acid supplements. Gal1 promoter-controlled genes were induced by growing cells in synthetic minimal medium containing 2% galactose as a carbon source. Growth was monitored by measuring absorbance at 600 nm (A_{600}).

Genetic techniques

Single step chromosomal tagging and deletion of genes were performed by homologous recombination as described by Longtine et al. [31]. Inserts for the construction of Myc- or HA-tagged genes were obtained by PCR from plasmids pFA6a-13Myc-*HIS3MX6* or pFA6a-*URA3KL* from *K. lactis*. Primers used for amplification of the respective DNA-fragments are listed in Table 2. For transformation of yeast strains, 300-600 ng DNA were used according to the high-efficiency lithium acetate transformation protocol [32]. Transformants were incubated on minimal medium lacking the respective amino acid for 3 days at 30°C. Positive transformants were verified for correct integration of the fusion cassette by colony-PCR of whole yeast cell extracts. For the construction of the pYES2-*DGA1* and pYES2-*ARE2* plasmid, the open reading frames of *DGA1* and *ARE2*, respectively, were amplified using genomic DNA from *S. cerevisiae* wild type as a template. Prior to ligation, the insert and the vector were cleaved by *BamH*I and *Eco*RI. Primers used for PCR are listed in Table 2.

Isolation and characterization of subcellular fractions

Yeast LD and microsomal fractions (30,000 x g) were isolated from cells grown to the stationary growth phase as described by Leber et al. [4] and Zinser et al. [33,34]. The quality of subcellular fractions was routinely tested by Western blot analysis.

Protein analysis

Proteins of isolated fractions were precipitated with trichloroacetic acid at a final concentration of 10% and solubilized in 100 µL 0.1% SDS, 0.1% NaOH. Samples of LD were delipitated prior to protein quantification. Non-polar lipids were extracted with 2-3 volumes of diethyl ether, the organic phase was withdrawn, and the sample was dried under a stream of nitrogen. Proteins were quantified by the method of Lowry et al. [35] using bovine serum albumin (BSA) as a standard. SDS-PAGE was carried out as described by Laemmli [36] using 12.5% separation gels; and Western blot analysis was performed as described by Haid and Suissa [37].

Preparation of total cell extracts for lipid analysis

For the preparation of total cell extracts, yeast cells were grown to the logarithmic or the stationary phase, respectively. Cells were harvested by centrifugation at 3,000 x g for 5 min at room temperature and suspended in distilled water. Then, glass beads were added and cells

were disintegrated by vigorous shaking on a vortex mixer for 10 min at 4°C. After cell disintegration, total cell extracts (homogenates) were used for lipid extraction.

Lipid analysis

Lipids from total cells or subcellular fractions were extracted following the procedure of Folch et al. [38] using chloroform/methanol (2:1; v/v) as solvent. For quantification of non-polar lipids, lipid extracts were applied to thin layer chromatography (TLC) plates (silica gel 60). Chromatograms were developed in an ascending manner (5 min) using light petroleum/diethyl ether/acetic acid (35:15:1; per vol.) as a solvent, briefly dried and further developed for 20 min using light petroleum/diethyl ether (49:1; v/v) as a second solvent system. To visualize separated bands, TLC plates were dipped into a solution consisting of 0.63 g $MnCl_2.4H_2O$, 60 mL water, 60 mL methanol, and 4 mL concentrated sulfuric acid; and incubated in a heating chamber at 105°C for at least 30 min. TG and SE bands were quantified by densitometric scanning at 400 nm using a CAMAG TLC Scanner 3. Triolein (Nu-Check Prep) and cholesteryl oleate (Sigma-Aldrich) served as standards.

The different classes of phospholipids were separated by 2-dimensional TLC using chloroform/methanol/25% ammonia (65:35:5; per vol.) as solvent system for the first dimension; and chloroform/acetone/methanol/acetic acid/water (50:20:10:10:5; per vol.) for the second dimension. Spots were visualized by staining with iodine vapor and scraped off, and lipid phosphorus was quantified by the method of Broekhuyse [39].

RNA isolation and Real-Time PCR

For RNA isolation, cells were grown to the mid-logarithmic growth phase on YPD at 30°C. Total RNA was isolated using an RNeasy kit from Qiagen following the manufacturer's instructions. After DNaseI digestion, Real-Time PCR was performed using the SuperScript III Platinum SYBR Green One-Step qRT-PCR Kit (Invitrogen) as described by the manufacturer. Amplification was measured by using an ABI 7500 instrument (Applied Biosystems) in sealed MicroAmp Optical 96-Well Reaction Plates. Relative quantities of RNA were determined using the ΔΔCt method described by Livak and Schmittgen [40]. Differences in mRNA expression were calculated after normalization with *ACT1* relative to wild type control. Primers used for RT-PCR are listed in Table 3.

***In vivo* labeling of total lipids**

For *in vivo* labeling of total lipids, wild type and *tgl3*Δ*tgl4*Δ*tgl5*Δ were inoculated to an A_{600} of 0.1 in 200 mL YPD and grown to the early logarithmic (A_{600} of ~1) or stationary growth phase (A_{600} of ~13). Aliquots of 10-20 mg cells were pre-incubated for 15 min at 30°C in 10 mL YPD. Then, 10 µL [1-^{14}C]oleic acid (0.1 mCi/mL; Perkin Elmer Life Sciences) or 20 µL [1,2-^{14}C]acetic acid (0.1 mCi/mL; Perkin Elmer Life Sciences), respectively, were added to the cells and incubated at 30°C for 20 and 30 min. Cells were harvested by centrifugation after adding 1 mL of 2% BSA. Then, cells were washed with a pre-cooled 0.5% BSA solution and distilled water. The cell pellet was resuspended in ice-cold water and disintegrated by vigorous shaking on a vortex mixer in the presence of glass beads for 10 min at 4°C. Lipids were extracted, separated by TLC as described above and visualized with iodine vapor. TG, SE and phospholipid bands were scraped off the plates, and radioactivity was measured by liquid scintillation counting using LSC Safety (Baker, Deventer, The Netherlands) with 5% water as a scintillation cocktail.

Enzyme assays

The acyl-CoA:diacylglycerol acyltransferase assay was performed in a final volume of 400 µL containing 0.7 nmol [^{14}C]oleoyl-CoA (58.6 mCi/mmol; PerkinElmer Life Sciences); 200 µM unlabeled oleoyl-CoA (Sigma-Aldrich); 12.5 µM diacylglycerol; 0.5 mM Tris-Cl, pH 7.0; 15 mM KCl; 15 mM $MgCl_2$; 0.25% TritonX100; 200 µg fatty acid free BSA; and 150 µg dithiothreitol. Homogenate (0.4 to 1 mg protein) or microsomes (0.2 to 0.6 mg protein) were used as enzyme sources. For measuring the phospholipid:diacylglycerol acyltransferase activity the 400 µL assay mixture contained 7.8 nmol [^{14}C]phospholipids (2400 dpm/nmol); 25 µM diacylglycerol; 0.5 mM Tris-Cl, pH 7.0; 15 mM KCl; 15 mM $MgCl_2$; 0.25% Triton X100; 200 µg BSA; and 150 µg dithiothreitol. Homogenate (0.4 to 1 mg protein) or microsomal fractions (0.2 to 0.6 mg protein) were used as enzyme sources. Radioactive phospholipid substrates were prepared as described by Horvath et al. [41]. Both assays were carried out at 30°C for 30 min.

The acyl-CoA:ergosterol acyltransferase assay was performed in a final volume of 400 µL containing 0.7 nmol [^{14}C]oleoyl-CoA (58.6 mCi/mmol; PerkinElmer Life Sciences); 50 µM unlabeled oleoyl-CoA (Sigma-Aldrich); 25 µM ergosterol; 0.5 mM CHAPS; 100 mM KPi, pH 7.4; 10 mM dithiothreitol; and 0.2 to 0.3 mg protein of the homogenate fraction, or 70 to 100 µg protein of a microsomal fraction as enzyme source. Reactions were carried out at 30°C for 10 min and stopped by the addition of 3 mL chloroform/methanol (2:1; v/v). Lipids

were extracted and separated by TLC as described above. Bands were scraped off the plate, and radioactivity incorporated into TG or SE was measured as described above.

Electron microscopy of yeast cells

For ultrastructural inspection, cells were grown under aerobic conditions at 30°C on YPD to the stationary growth phase. Cells were washed three times with double-distilled water. Subsequently, cells were fixed for 5 min in a 1% aqueous solution of $KMnO_4$ at room temperature, washed with distilled water, and fixed again in a 1% aqueous solution of $KMnO_4$ for 20 min. Fixed cells were washed three times with distilled water and incubated in 0.5% aqueous uranylacetate overnight at 4°C. Samples were dehydrated in a graded series of acetone (50, 70, 90, and 100%) and gradually infiltrated with increasing concentrations of Spurr resin (30, 50, 70, and 100%) mixed with acetone for a minimum of 3 h per step. Samples were finally embedded in pure, fresh Spurr resin and polymerized at 60°C for 48 h. Ultrathin sections of 80 nm were stained with lead citrate and viewed with a Philips CM 10 electron microscope.

Results

Non-polar lipid analysis of yeast cells bearing a defect in TG hydrolysis

Tgl3p, Tgl4p and Tgl5p are the major TG lipases in the yeast *S. cerevisiae* [24,25]. Athenstaedt and Daum (25) showed that a *tgl3*Δ*tgl4*Δ*tgl5*Δ triple mutant (TM) lacking the major TG lipases accumulated TG at high amount, but also SE. Based on these findings we investigated the metabolic effect of TG hydrolysis on TG and SE synthesis.

TG and SE accumulate in the stationary growth phase of a yeast culture when LD biogenesis is prominent. After 24 h of growth (stationary phase) the relative amount of TG in cells lacking TG lipases (TM) was strongly increased (~200%) over wild type (Fig. 1A). However, a similar effect was also observed with the TM grown to the logarithmic growth phase (10h), although the total amount of TG formed was markedly lower. The increased TG content of the TM in both growth phases can be explained by the lack of lipolytic activity rendering strains largely unable to mobilize TG. A different effect of the *tgl3*Δ*tgl4*Δ*tgl5*Δ triple mutation was observed for SE synthesis. As already shown before [25] the level of SE was increased in the TM grown to the stationary growth phase (Fig. 1B, 24h). In contrast, the triple mutation had only a moderate effect on the level of SE in the logarithmic growth phase.

These data indicate that the block of TG degradation and the resulting SE accumulation are growth phase dependent.

To test whether phospholipid metabolism was also affected by the accumulation of non-polar lipids we quantified total phospholipids in wild type and TM grown to the logarithmic and stationary growth phase, respectively (Fig. 1C). The total amount of phospholipids was only slightly affected by the triple mutation and rather decreased in the logarithmic growth phase (Fig. 1C, 10h). In the stationary growth phase, however, the level of phospholipids was slightly increased in the TM (Fig. 1C, 24h), although the phospholipid composition was not affected (data not shown). This result indicates, that also phospholipid metabolism responds at least to some extent to non-polar lipid accumulation in the TM.

To investigate the individual contributions of the TG synthases Dga1p and Lro1p to TG and SE accumulation in the TM background we quantified non-polar lipids in the respective mutant strains. As mentioned before, Dga1p was reported to be the more active TG synthase in yeast cells grown to the stationary phase [14]. To test this hypothesis, we performed TG and SE analyses of *tgl3*Δ*tgl4*Δ*tgl5*Δ with an additional deletion of *DGA1* or *LRO1*, respectively (Fig. 2A and B). TG levels of TM*dga1*Δ and TM*lro1*Δ were markedly lower than in *tgl3*Δ*tgl4*Δ*tgl5*Δ, although the amounts of TG were still increased in both strains compared to wild type (Fig. 2A). However, Dga1p seems to be the major contributor to TG synthesis in the stationary growth phase also in the TM because deletion of *DGA1* in the TM background led to a marked decrease of TG. In contrast, deletion of *LRO1* caused only a moderate change of the TG level. Interestingly, deletion of either *DGA1* or *LRO1* reduced the total amount of SE in the TM back to the wild type level (Fig. 2B). This result indicates that the presence of both TG synthases is required for SE accumulation in the TM.

In a complementary approach, we tested the effect of *DGA1* and *ARE2* overexpression on non-polar lipid formation in *tgl3*Δ*tgl4*Δ*tgl5*Δ. For the overexpression of the respective inducible genes, cells were cultivated in synthetic minimal medium with galactose as carbon source (see Material and Methods). Under these conditions, TG and SE accumulation in the TM was even more pronounced than during standard cultivation (Figs 2C and D). Overexpression of *DGA1* alone in the wild type background increased the TG level up to ~250% of the control (Fig. 2C), and overexpression of *ARE2* caused a markedly enhanced SE formation (Fig. 2D). Most interestingly, overexpression of *DGA1* in the TM did not further increase the total amount of TG suggesting a limited capacity of yeast cells to form this storage lipid. Surprisingly, SE formation was slightly enhanced under these conditions. In contrast, overexpression of *ARE2* resulted in a shift of non-polar lipid synthesis from TG to

SE (see Figs 2C and D). These findings can be explained by the fact that both Dga1p and Are2p use acyl-CoAs for acylation reaction and thus compete for these substrates. The shift in non-polar lipid formation can be best documented by the TG to SE ratios in the respective strains (Table 4). Whereas this ratio was similar in the TM and wild type, it was decreased in the TM when *DGA1* and *ARE2* were overexpressed. These results were slightly different from previously published data [25] due to differences in experimental procedures.

Although lipid storage is not essential for the yeast [9], overproduction of storage lipids seems to influence cell viability negatively. The TM bearing the empty vector control showed already reduced viability when cultivated on minimal medium with galactose as carbon source (Fig. 3). This effect was also observed when cells were grown on glucose as carbon source, although less pronounced. Overexpression of *ARE2* in the TM caused a marked growth defect. Interestingly, also an overexpression of *ARE2* in wild type background led to a growth defect as demonstrated before by global phenotypic analysis [42,43]. Furthermore overexpression of *DGA1* in the TM grown on galactose reduced cell viability dramatically. A loss of viability was already observed without induction of the gene expression with glucose as carbon source. The reason for this observation may be that the Gal1 promotor was not completely tight. Taken together, the increased level of TG combined with the elevated level of SE seems to have a negative effect on cell viability.

Characterization of LD from yeast cells lacking TG lipases

To investigate the effect of the above mentioned changes in the TG and SE content of a *tgl3*Δ*tgl4*Δ*tgl5*Δ strain on structural properties of the cell, we analyzed LD by electron microscopy. This analysis revealed an increased size and number of LD in *tgl3*Δ*tgl4*Δ*tgl5*Δ (Fig. 4). LD from wild type contained 25 ± 6.1 µg TG/µg protein and 18.5 ± 2.9 µg SE/µg protein confirming previous results from our laboratory [4]. In cells lacking TG lipases, the TG content of LD was increased to 41.4 ± 13.7 µg/µg protein, and the SE content to 28.7 ± 8.4 µg/µg protein. Thus, both non-polar lipids were enriched in LD ~1.7-fold over wild type. These enrichment factors reflect the pattern of non-polar lipids in total cell extracts (see Fig. 1). We also observed a slight increase in the phospholipid content of LD from cells lacking TG lipases. LD from wild type contained 1.05 ± 0.09 µg phospholipid/µg protein, whereas in LD from *tgl3*Δ*tgl4*Δ*tgl5*Δ the phospholipid amount was increased to 1.23 ± 0.18 µg/µg protein. The phospholipid composition of LD, however, was similar in mutant and wild type with phosphatidylcholine representing the most abundant phospholipid class followed by phosphatidylinositol and phosphatidylethanolamine (data not shown)

***In vivo* labeling of lipids in cells bearing a defect in TG mobilization**

Lipid analysis of total cell extracts and LD revealed a balance between TG and SE synthesis, as both non-polar lipids accumulated when TG hydrolysis was blocked. To shed more light on TG and SE synthesis under these conditions, *in vivo* labeling of lipids in wild type and *tgl3*Δ*tgl4*Δ*tgl5*Δ was performed. Fig. 5A shows the incorporation of [^{14}C]oleic acid into TG, SE and phospholipids from wild type and TM in the logarithmic growth phase. Most of the radiolabeled oleic acid was incorporated into phospholipids (~80%), whereas only minor amounts of the label ended up in TG and SE (approximately 10%, each). In *tgl3*Δ*tgl4*Δ*tgl5*Δ, *in vivo* labeling of all measured lipid classes was similar to wild type. This result demonstrates that non-polar lipid synthesis was not much affected upon a block of TG degradation in the logarithmic growth phase.

Fig. 5B shows short time *in vivo* labeling of lipids with [^{14}C]oleic acid when wild type and the TM reached the early stationary growth phase. Under these conditions only ~50% of the label was incorporated into phospholipids, and the other half of the label was used for the synthesis of TG and SE. In wild type, TG and SE were formed at nearly equal amounts, but in the *tgl3*Δ*tgl4*Δ*tgl5*Δ strain the labeling pattern of non-polar lipids was markedly changed. In cells lacking TG lipases, incorporation of [^{14}C]oleic acid into TG was decreased, whereas labeling of SE and phospholipids was slightly enhanced. Thus, in the phase of strong TG accumulation its formation was blocked in a kind of feedback regulation. Increased synthesis of SE and phospholipids appears to be a means to get rid of fatty acids accumulated in *tgl3*Δ*tgl4*Δ*tgl5*Δ. Incorporation of [^{14}C]oleic acid into different classes of phospholipids in the TM was similar to wild type (Fig. 5C), indicating a rather unspecific effect caused by the excess of fatty acids. It has to be mentioned that the total incorporation of [^{14}C]oleic acid into lipids from cells lacking TG lipases was slightly increased compared to wild type in both growth phases. Values of 12,300 ± 1,600 (wild type) and 14,100 ± 2,200 cpm/mg cell wet weight (TM) were obtained for cells grown to the logarithmic phase; and 11,600 ± 2,500 (wild type) and 14,600 ± 3,210 cpm/mg cell wet weight (TM) for cells grown to the stationary phase. This result suggests that the overall formation of complex lipids was rather stimulated when TG hydrolysis was blocked.

Similar results as described above were obtained when [^{14}C]acetic acid was used as lipid precursor (Figs 6A and B). In the logarithmic growth phase, lipid formation in the TM was similar to wild type, whereas in the stationary growth phase incorporation of acetic acid into TG from TM was reduced. In contrast, incorporation of acetic acid into phospholipids was slightly increased in the mutant. These data confirmed the metabolic link between membrane

lipid and storage lipid synthesis. However, we also determined incorporation of [^{14}C]acetic acid into total fatty acids to test a possible influence of the *tgl3*Δ*tgl4*Δ*tgl5*Δ triple mutation on fatty acid biosynthesis. In the logarithmic growth phase incorporation of [^{14}C]acetic acid into lipids was only moderately changed and rather decreased in the mutant (data not shown). In the stationary growth phase incorporation of [^{14}C]acetic acid into lipids from the TM (1,460 ± 160 cpm/mg cell wet weight) was similar to wild type (1,300 ± 100 cpm/mg cell wet weight). These data indicate that fatty acid production was not inhibited by the triple mutation, and lack of TG lipases did not cause a feedback regulation of fatty acid production.

Gene expression, protein levels and enzymatic activities of non-polar lipid synthesizing enzymes in cells lacking TG lipases

Changes in the lipid pattern caused by the *tgl3*Δ*tgl4*Δ*tgl5*Δ triple deletion raised the question as to the molecular reason for this finding. In principle, the triple mutation could affect gene expression, the protein levels, and/or enzymatic activities of non-polar lipid synthesizing enzymes. Gene expression analysis of *DGA1, LRO1, ARE1* and *ARE2* was performed with Real-Time PCR, and protein levels of TG and SE synthases were tested by Western Blot analysis. Surprisingly, neither gene expression (data not shown) nor protein levels (Fig. 7) of the four acyltransferases were changed in the TM compared to wild type.

To test whether a block of TG degradation and the resulting TG accumulation affected the activity of non-polar lipid synthesizing enzymes, we performed *in vitro* enzyme assays of Dga1p, Lro1p, Are1p and Are2p with isolated subcellular fractions. ER and LD fractions were purified from wild type and TM grown to the stationary growth phase and tested for TG and SE synthase activity, respectively. As can be seen from Table 5, the Dga1p activity in total cell extracts from TM was only slightly changed and rather enhanced compared to wild type. In microsomes of the TM, however, the enzymatic activity of Dga1p was markedly lowered, whereas it was increased in LD. The activity of Lro1p was slightly reduced (~15%) in both homogenate and in the microsomal fraction from TM. It has to be mentioned, however, that the absolute specific activity of Lro1p (41.5 pmol TG formed/mg protein/h) was low compared to Dga1p (214.2 pmol TG formed/mg protein/h) in the homogenate of wild type confirming previous results with wild type and TM grown to the stationary growth phase [14]. In contrast to TG synthase activity, SE synthase activity was slightly increased (15-20%) in both the homogenate and the microsomal fraction from cells lacking TG lipases. As gene expression and protein levels of non-polar lipid synthesizing enzymes were not

altered in *tgl3*Δ*tgl4*Δ*tgl5*Δ, changes in the TM were mainly caused by regulation at the enzymatic level.

Discussion

Non-polar lipid metabolism is a complex network of reactions with different enzymes involved [9,25]. Although catabolic as well as anabolic pathways of TG and SE metabolism were studied in some detail, the knowledge about the cross-talk of these pathways is still limited. Therefore, the present study was initiated to shed some light on possible metabolic links between non-polar lipid synthesis and degradation in the yeast *S. cerevisiae*.

Non-polar lipid analyses and *in vivo* labeling of lipids with wild type and *tgl3*Δ*tgl4*Δ*tgl5*Δ demonstrated that TG and SE metabolism are indeed inseparably linked processes. A block of TG hydrolysis caused by deletion of genes encoding the major TG lipases led to reduced TG synthesis, whereas SE formation was enhanced. In the former case, a feedback inhibition in the TG metabolic pathway seems plausible, whereas enhanced SE synthesis may be regarded as a way out to solve the problem of fatty acid accumulation, as Dga1p, Are1p and Are2p use the same substrate for acylation. Surprisingly, a further possible feedback control of TG formation in the TM, inhibition of fatty acid synthesis, was not observed. Actually, the unaffected fatty acid synthesis in combination with the lipolytic defect in the TM appears to be the reason for TG accumulation in this strain.

As transcription of *DGA1* and *LRO1* as well as the levels of the respective gene products were similar in wild type and *tgl3*Δ*tgl4*Δ*tgl5*Δ, the possibility remained that TG and also SE synthases were subject to regulation on the enzymatic level. In microsomes (endoplasmic reticulum), which are considered as the major site of non-polar lipid formation, a decrease in the activity of TG synthases was observed, whereas SE synthase activity was increased (see Table 5). These results are in line with the observed changes in product formation of the triple mutant (see Fig. 1). The puzzling observation, however, was the increased Dga1p activity *in vitro* measured with LD samples, which may be the reason for enhanced Dga1p activity *in vitro* in total cell extracts of TM compared to wild type (see Table 5). The discrepancy of the enzyme analysis versus labeling experiments and quantification of lipid products can be explained by the different situation *in vitro* and *in vivo.* In the case of Dga1p, we have to keep in mind that despite the marked activity of the enzyme measured with isolated LD the physiological relevance of Dga1p in this compartment remains elusive. Besides their role in non-polar lipid storage and turnover, LD were discussed as a storage

compartment for protein aggregates and incorrectly folded proteins [8,44]. It was also suggested that LD may be a temporary storage site for proteins on their way to degradation [45], or a depot of proteins that may be released and reutilized upon requirement [46,47]. Thus, LD may play a role in managing the availability of proteins, and Dga1p might not even be active *in vivo* when located to LD. The membrane topology of Dga1p might play a critical role for its activity. When located to the ER, Dga1p is inserted into a phospholipid bilayer, whereas on LD Dga1p needs to cope with a phospholipid monolayer environment which might not be favorable for the activity of the enzyme. The topology of Dga1p inserted into the ER membrane was recently investigated [48]. Specific domains of the protein were identified which face the ER lumen and play an essential role in enzyme catalysis. The topology of Dga1p on LD was not yet determined, and therefore a possible effect of the membrane environment on the activity of Dga1p in LD remains speculative at present.

The observation that TG and SE synthases are subject to regulation on the enzymatic level (see Table 6) leaves us with the question as to possible regulatory mechanisms involved. Information about this aspect is rare, although evidence for post-translational modifications of Dga1p has been presented [49,50]. Overexpression of *DGA1* in *snf2*Δ, a strain deleted of the catalytic subunit of the SWI/SNF chromatin remodeling complex, markedly increased diacylglycerol acyltransferase activity, although the protein level of Dga1p was not changed [51]. Furthermore, a global analysis of protein phosphorylation in yeast [52] identified Dga1p as target of the protein kinase Kss1p. Similarly, the SE synthase Are2p seems to be target of various protein kinases in the yeast [52]. We have to keep in mind, however, that these results are based on a global phosphorylation screening which requires individual verification and further elucidation. Another, although completely speculative possibility may be that the activities of TG and SE synthases are modulated by binding partner proteins. A prominent example of his kind is the interaction of the mammalian TG lipase ATGL with CGI-58, which acts as an important activator [53,54]. So far, however, no such partner proteins have been identified for yeast TG lipases.

As already mentioned above, the *tgl3*Δ*tgl4*Δ*tgl5*Δ triple deletion leads to growth phase dependent changes in phospholipid formation. Whereas in the exponential growth phase the level and the synthesis of phospholipids seems to be slightly reduced in the TM, a slight increase was observed in cells grown to the stationary phase (see Figs 1, 5 and 6). Changes in the phospholipid level were moderate and did not result in enhanced membrane formation as can be judged from electron microscopy (see Fig. 4). The opposite trend of phospholipid formation in the two different growth phases may be result of the different enzymatic

activities which had been attributed to Tgl3p, Tgl4p and Tgl5p. Previous results from our laboratory had demonstrated that the three TG lipases do not only act as hydrolytic enzymes but also contribute to phospholipids synthesis as lysophospholipid acyltransferases [28,29]. Rajakumari et al. [27] demonstrated that TG lipolysis is linked to sphingolipid and phospholipid metabolism. These experiments, however, were obtained with a *tgl3*Δ*tgl4*Δ*tgl5*Δ strain grown to the early logarithmic growth phase. In this phase, where membrane formation is a major issue, the acyltransferase activities of Tgl3p, Tgl4p and Tgl5p may be more relevant than in the stationary growth phase, where lipid storage is predominant. In the latter situation, the overflow of fatty acids may lead to the observed moderate overproduction of phospholipids.

Taken together, a number of regulatory links within the lipid metabolic network seem to be involved in cellular lipid homeostasis which we are just beginning to understand. A recent study from our own laboratory [55] addressed such a regulatory link, namely the effect of non-polar lipid formation on the behavior of TG lipases in the yeast. Thus, storage of lipids in the form of TG and SE and its regulation appear to be important for the lipid metabolic network by providing some flexibility to the cell with respect to energy consumption, membrane and organelle formation, and viability.

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Abbreviations: BSA, bovine serum albumin; E, ergosterol; ER, endoplasmic reticulum; LD, lipid droplets; PL, phospholipids; SE, steryl esters; TG, triacylglycerols; TLC, thin layer chromatography; TM, triple mutant.

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Tables

Table1 Yeast strains used in this study

Strain	Genotype	Source
Wild type (WT)	BY4741 *Mat a; his3Δ1; leu2Δ0; met15Δ0; ura3Δ0*	Euroscarf
*tgl3*Δ*tgl4*Δ*tgl5*Δ (TM)	BY4741; *tgl3*Δ::*kanMX4; tgl4*Δ::*kanMX4; tgl5*Δ::*kanMX4*	[25]
*tgl3*Δ*tgl4*Δ*tgl5*Δ*dga1*Δ	See TM; *dga1*Δ::*URA3KL*	This study
*tgl3*Δ*tgl4*Δ*tgl5*Δ*lro1*Δ	See TM; *lro1*Δ::*URA3KL*	This study
Dga1p-HA	See WT; *DGA1-HA*::*URA3KL*	This study
*tgl3*Δ*tgl4*Δ*tgl5*Δ Dga1p-HA	See TM; *DGA1-HA*::*URA3KL*	This study
Lro1p-Myc	See WT; *LRO1-13Myc::HIS3MX6*	This study
*tgl3*Δ*tgl4*Δ*tgl5*Δ Lro1p-Myc	See TM; *LRO1-13Myc::HIS3MX6*	This study
Are1p-Myc	See WT; *ARE1-13Myc*::*HIS3MX6*	Kindly provided by M. Korber
*tgl3*Δ*tgl4*Δ*tgl5*Δ Are1p-Myc	See TM; *ARE1-13Myc*::*HIS3MX6*	This study
Are2p-Myc	See WT; *ARE2-13Myc*::*HIS3MX6*	Kindly provided by M. Korber
*tgl3*Δ*tgl4*Δ*tgl5*Δ Are2p-Myc	See TM; *ARE2-13Myc*::*HIS3MX6*	This study

Table 2 Primers used throughout this study

Primer	Sequence (5'→3')
Dga1HAfwd	TACGAAAATAGAGAAAAATATGGGGTACCGGATGCAGAATTGAAGATAGTTGGGTACCCATACGATGTTCCTGACTATGCGTAACGGATCCCCGGGTTAATTAA
Dga1HArev	TAAAAAATCCTTATTTATTCTAACATATTTTGTGTTTTCCAATGAATTCATTATTAATCGATGAATTCGAGCTCG
Lro1Mycfwd	GCCAATTGTCTAATTTGAGCCAGTGGGTTTCTCAGATGCCCTTCCCAATGCGGATCCCCGGGTTAATTAA
Lro1Mycrev	TCTTTTCGCTCTTTGAAATAATACACGGATGGATAGTGAGTCAATGTCGGTCATTTAATCGATGAATTCGAGCTCG
Are1Mycfwd	TTGGTGTCTGTTCAGGGCCCAGTATCATTATGACGTTGTACCTGACCTTACGGATCCCCGGGTTAATTAA
Are1Mycrev	TTGTATATCTATCAAGGGCTTGCGAGGGACACACGTGGTATGGTGGCAGTATCGATGAATTCGAGCTCG
Are2Mycfwd	TCGGTATCTGCATGGGACCAAGTGTCATGTGTACGTTGTACTTGACATTCCGGATCCCCGGGTTAATTAA
Are2Mycrev	AAAATTTACTATAAAGATTTAATAGCTCCACAGAACAGTTGCAGGATGCCATCGATGAATTCGAGCTCG
Dga1delfwd	TACATATACATAAGGAAACGCAGAGGCATACAGTTTGAACAGTCACATAAATGCGTACGCTGCAGGTCGAC
Dga1delrev	TAAAAAATCCTTATTTATTCTAACATATTTTGTGTTTTCCAATGAATTCATTATTAATCGATGAATTCGAGCTCG
Lro1delfwd	AGCCATTACAAAAGGTTCTCTACCAACGAATTCGGCGACAATCGAGTAAAAAATGCGTACGCTGCAGGTCGAC
Lro1delrev	TCTTTTCGCTCTTTGAAATAATACACGGATGGATAGTGAGTCAATGTCGGTCATTTAATCGATGAATTCGAGCTCG
Dga1Bamfwd	TATAGGATCCATGTCAGGAACATTCAATGATATAA
Dga1Ecorev	TATAGAATTCTTACCCAACTATCTTCAATTCTG
Are2Bamfwd	TATAGGATCCATGGACAAGAAGAAGGATCTACT
Are2Ecorev	TATAGAATTCTTAGAATGTCAAGTACAACGTACA

Table 3 Primers used for RT-PCR

Primer	Sequence (5'→3')
RTAct1fwd	CCAGCCTTCTACGTTTCCATCCAAG
RTAct1rev	GACGTGAGTAACACCATCACCGGA
RTDga1fwd	GGTGGCGCTAGGGAATCTTTATTAAG
RTDga1rev	AGGTTAATATTCCCCGTTTGAATGGCC
RTLro1fwd	TCACCTGAATGGCTCCAAAGAAGAGT
RTLro1rev	GACCAGTGCTTGTGGTGTAGCTCA
RTAre1fwd	CCACCTGCTCTTCGACATGATTCC
RTAre1rev	GGTCAACTCCGCCACGCAATTCAA
RTAre2fwd	TATCCTGTAGCAATGAGAGCATTGGCT
RTAre2rev	ATAAACCCTGGGACGATATCAACGAG

Table 4 Non-polar lipid analysis from wild type (WT), *tgl3*Δ*tgl4*Δ*tgl5*Δ (TM) and TM overexpressing *DGA1* (TM*DGA1*) or *ARE2* (TM*ARE2*), respectively. The ratios of triacylglycerols (TG) to steryl esters (SE) are shown. Data are mean values of at least three independent experiments with an error of ±15%.

	TG/SE
WT	1.4
TM	1.4
TM*DGA1*	1
TM*ARE2*	0.96

Table 5 Activities of non-polar lipid-synthesizing enzymes *in vitro* from wild type (WT) and *tgl3*Δ*tgl4*Δ*tgl5*Δ (TM). Data are mean values of at least three independent experiments with an error of ±15%. The specific activity of acyl-CoA:diacylglycerol acyltransferase was 214.2 pmol TG/mg protein/h; of phospholipid:diacylglycerol acyltransferase 41.5 pmol TG/mg protein/h; and of acyl-CoA:ergosterol acyltransferase 1035 pmol SE/mg protein/h. The specific enzymatic activities in the homogenate of wild type were set at 1. n.d., not detected.

		Relative enzymatic activities		
		Homogenate	Microsomes	Lipid droplet
Dga1p	WT	1	4.6	188.8
	TM	1.1	3.5	278.4
Lro1p	WT	1	7.9	n.d.
	TM	0.9	6.8	n.d.
Are1p/Are2p	WT	1	4.5	n.d.
	TM	1.2	5.3	n.d.

FIGURE 1

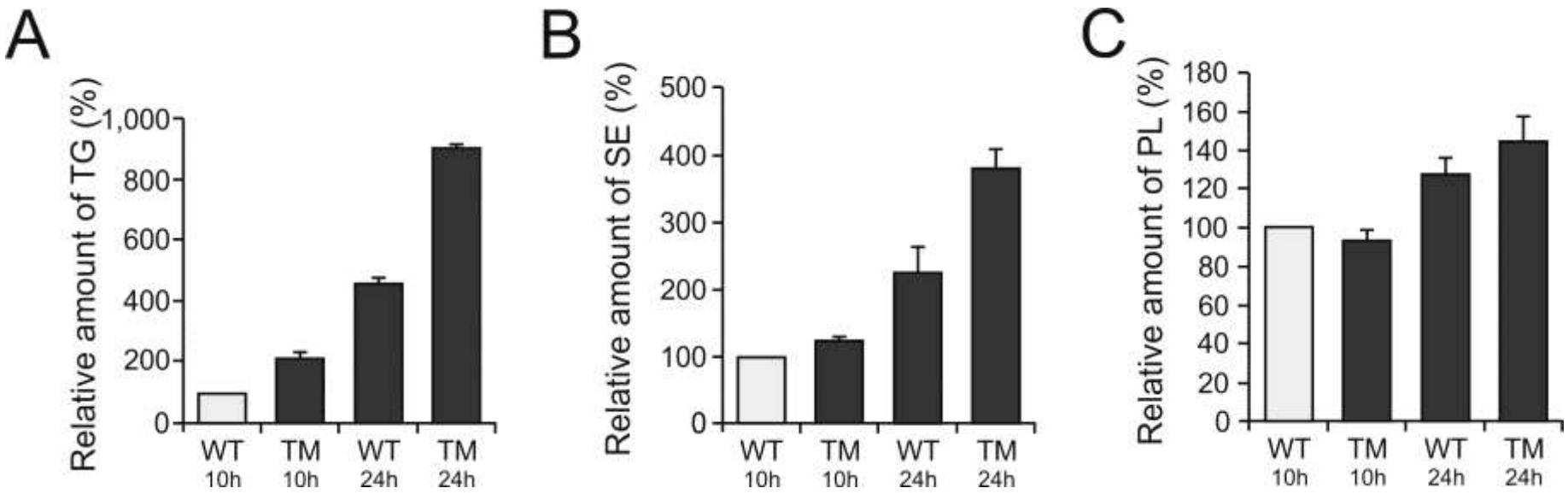

Fig. 1 Lipid analysis of wild type (WT) and *tgl3*Δ*tgl4*Δ*tgl5*Δ (TM). Relative amounts of triacylglycerols (TG) (A); steryl esters (SE) (B); and phospholipids (PL) (C) from cells grown to the early logarithmic (10 h) and stationary growth phase (24 h) were measured. The amounts of TG, SE and PL per A_{600} from WT in the logarithmic growth phase were set at 100%. Data are mean values of at least three independent experiments with the respective deviation.

FIGURE 2

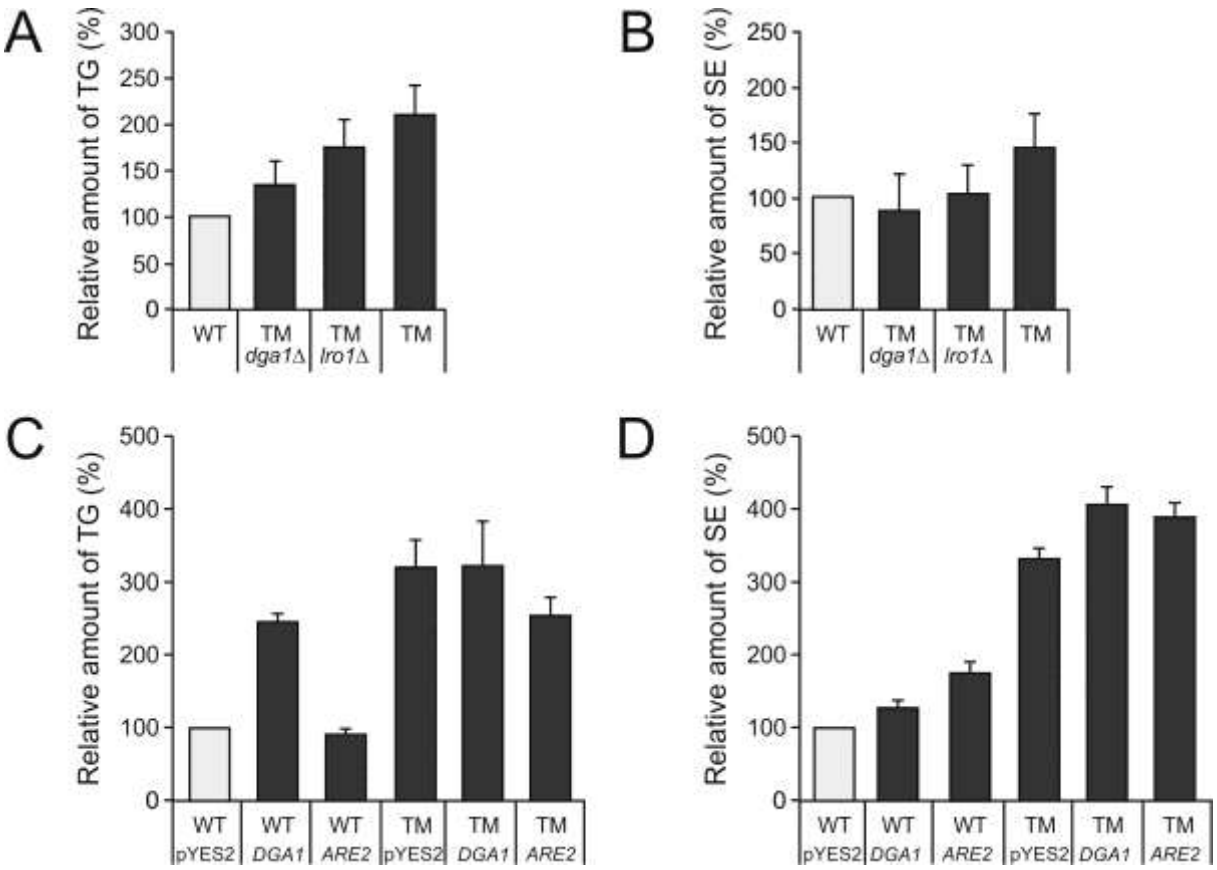

Fig. 2 Non-polar lipid analysis of yeast strains bearing a defect in triacylglycerol hydrolysis. (A) Relative amounts of triacylglycerol (TG) and (B) steryl esters (SE) from wild type (WT), *tgl3*Δ*tgl4*Δ*tgl5*Δ (TM), TM*dga1*Δ and TM*lro1*Δ grown to the stationary growth phase under standard cultivation condition were measured. (C) Relative amounts of TG and (D) SE from WT and TM bearing pYES2 (empty vector control), pYES2-*DGA1* or pYES2-*ARE2*, respectively, are shown. Cells were grown to the stationary growth phase on synthetic minimal medium containing galactose for induction of gene expression. The amounts of TG and SE per A_{600} from WT were set at 100%. Data are mean values of at least three independent experiments with the respective deviation.

FIGURE 3

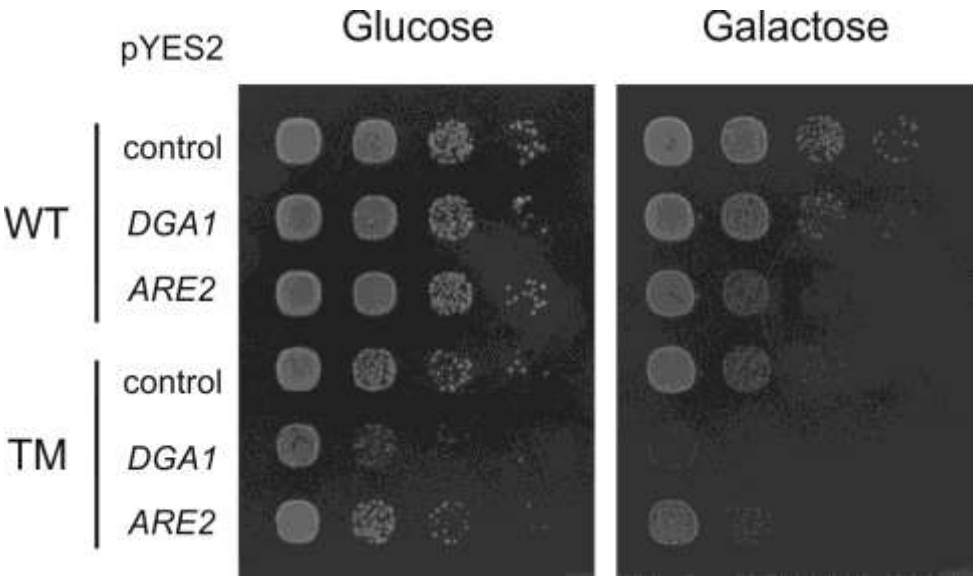

Fig. 3 Growth of yeast cells bearing a defect in triacylglycerol hydrolysis. Wild type (WT) and *tgl3*Δ*tgl4*Δ*tgl5*Δ (TM) bearing pYES2 (empty vector control), pYES2-*DGA1* or pYES2-*ARE2*, respectively, were spotted at dilutions of 1, 1/10, 1/100, 1/1000 on minimal medium with glucose or galactose as carbon source.

FIGURE 4

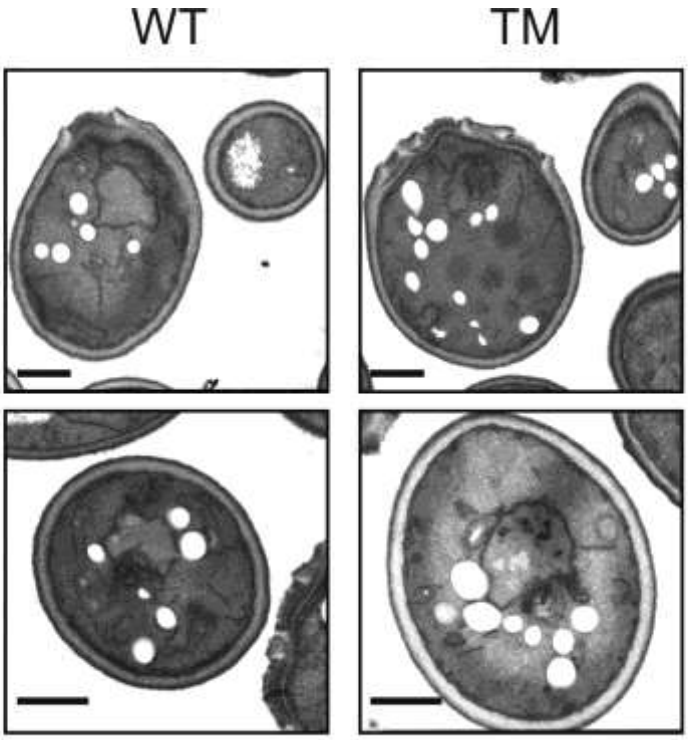

Fig. 4 Lipid droplets in cells bearing a defect in triacylglycerol degradation. Electron micrographs of wild type (WT) and *tgl3*Δ*tgl4*Δ*tgl5*Δ (TM) were prepared as described under "Matials and Methods". Size bar = 1μm.

FIGURE 5

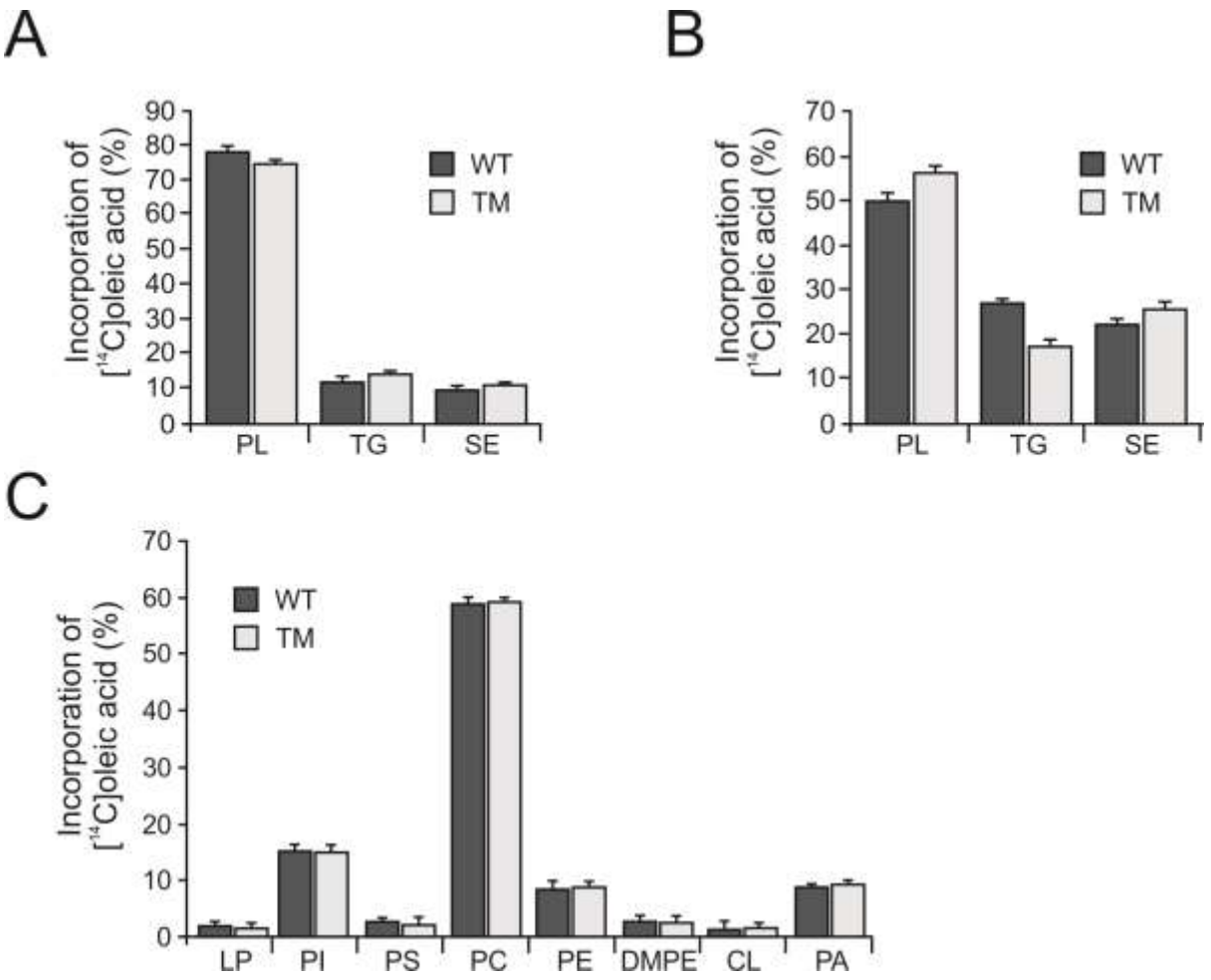

Fig. 5 *In vivo* labeling of lipids from cells lacking triacylglycerol lipases with [^{14}C]oleic acid. Relative incorporation of [^{14}C]oleic acid into steryl esters (SE), triacylglycerols (TG) and phospholipids (PL) from wild type (WT) and *tgl3*Δ*tgl4*Δ*tgl5*Δ (TM) at the (A) early logarithmic and (B) early stationary growth phase is shown. (C) The relative distribution of [^{14}C]oleic acid incorporated into lysophospholipids (LP), phosphatidylinositol (PI), phosphatidylserine (PS), phosphatidylcholine (PC), phosphatidylethanolamine (PE), dimethylphosphatidylethanolamine (DMPE), cardiolipin (CL) and phosphatidic acid (PA) in WT and TM is shown. Data are mean values of two experiments performed in duplicate with the respective deviation.

FIGURE 6

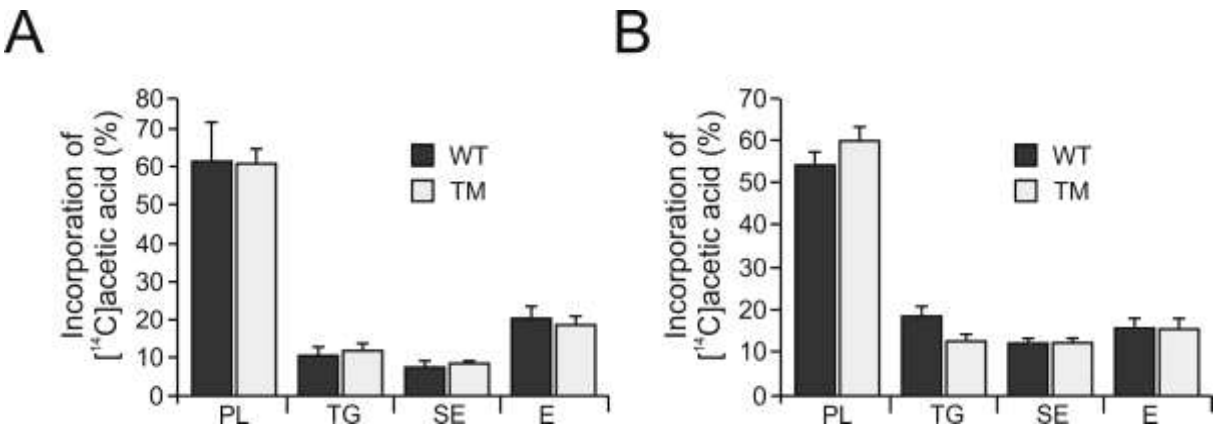

Fig. 6 *In vivo* labeling of lipids from cells lacking triacylglycerol lipases using [^{14}C]acetic acid. The relative incorporation of [^{14}C]acetic acid into phospholipids (PL), ergosterol (E), triacylglycerols (TG) and steryl esters (SE) in wild type (WT) and *tgl3*Δ*tgl4*Δ*tgl5*Δ (TM) which reached the (A) early logarithmic or (B) early stationary growth phase is shown. Data are mean values of two experiments performed in duplicate with the respective deviation.

FIGURE 7

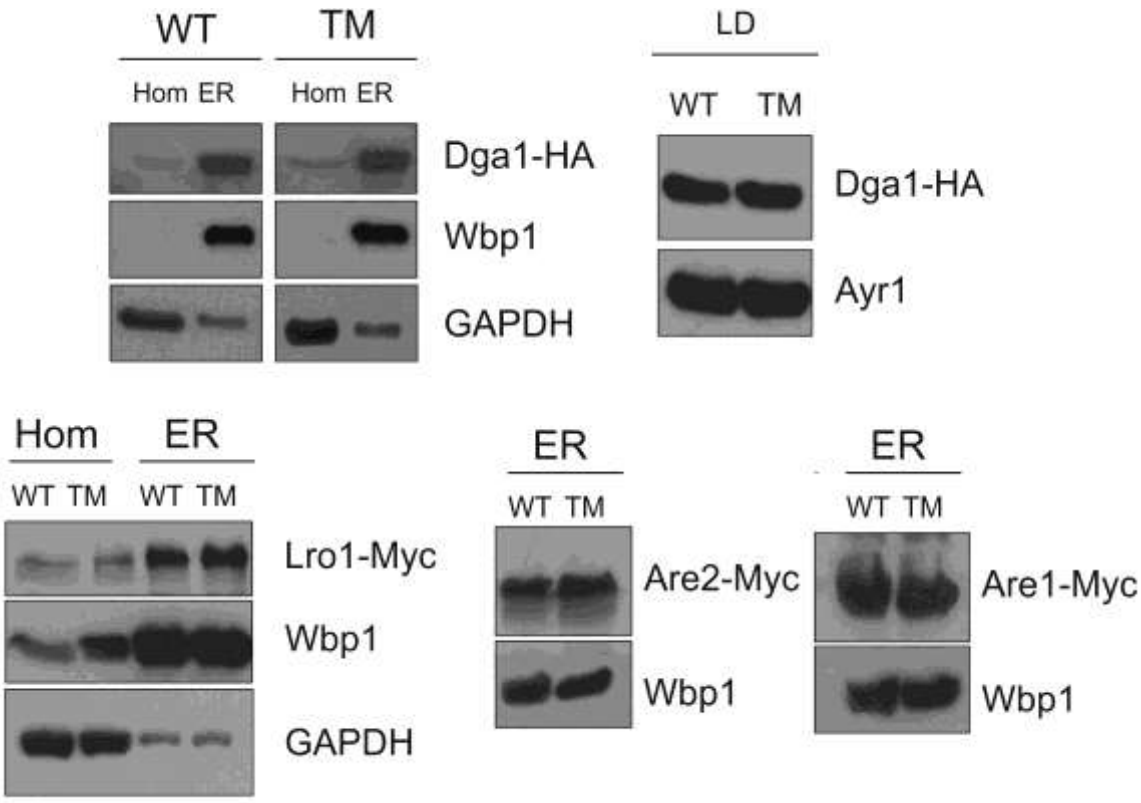

Fig. 7 Protein levels of triacylglycerol synthases and steryl ester synthases in cells lacking triacylglycerol lipases. Protein levels of Dga1-HA, Lro1-Myc, Are1-Myc and Are2-Myc were tested in wild type (WT) and *tgl3*Δ*tgl4*Δ*tgl5*Δ (TM) by Western Blot analysis. Homogenate (Hom), endoplasmic reticulum (ER) and lipid droplet (LD) fractions are shown. Antibodies were directed against HA-tag or Myc-tag, Wbp1 (ER marker), Ayr1 (LD marker) and GAPDH (cytosolic marker).

CHAPTER V

Regulation of triacylglycerol lipases Tgl4p and Tgl5p by presence/absence of non-polar lipids

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Keywords: Lipase, acyltransferase, triacylglycerol, lipid droplet, endoplasmic reticulum, yeast

Abstract

Tgl3p, Tgl4p and Tgl5p are the major triacylglycerol lipases of the yeast *S. cerevisiae*. Recent studies from our laboratory demonstrated that protein level, stability, localization and consequently activity of the major yeast triacylglycerol lipase Tgl3p are regulated by the formation of non-polar lipids (Schmidt et al., 2013, *J. Biol. Chem.* **288**, 19939–19948). The present study complements these investigations addressing the effect of non-polar lipid formation on the two other triacylglycerol lipases in the yeast, Tgl4p and Tgl5p. In contrast to Tgl3p, Tgl4p and Tgl5p are found at substantial amounts not only on lipid droplets, but also in the endoplasmic reticulum of wild type cells. Interestingly, protein level and abundance of Tgl4p and Tgl5p in the endoplasmic reticulum are hardly affected by the absence of non-polar lipids in a *dga1Δlro1Δare1Δare2Δ* quadruple mutant lacking all enzymes of non-polar lipid formation, although in this mutant strain, which is devoid of lipid droplets, the portions of Tgl4p and Tgl5p associated with this organelle are missing. Tgl4p and Tgl5p, which exhibit only minor lipolytic activity, catalyze also lysophospholipid acyltransferase activities. These activities seem to be preserved when Tgl4p and Tgl5p are retained in the endoplasmic reticulum of a quadruple mutant. These findings shed some new light on the non-polar lipid metabolic network of the yeast and provide first hints for links within the wide spreading lipid metabolic network in this microorganism.

Introduction

Accumulation of free fatty acids and sterols can lead to lipotoxicity resulting in defects in organelle membrane formation and cell integrity. Hence, cells have developed mechanisms to store these lipids in their inert forms, triacylglycerols (TG) and steryl esters (SE), respectively, in defined organelles called lipid droplets (LD) [1,2]. In the yeast which is used as a model system for our studies LD contain approximately 50% TG and 50% SE, each. LD are surrounded by a monolayer membrane of phospholipids with certain proteins associated or embedded [3–5].

Yeast TG provide a source of fatty acids for membrane biogenesis. They are formed by two different pathways governed by Dga1p (acyl-CoA:diacyglycerol acyltransferase) and Lro1p (phospholipid:diacylglycerol acyltransferase) [6–9]. The yeast enzyme Dga1p belongs to the diacylglycerol acyltransferase gene family DGAT2 and can form TG from DG and acyl-CoAs as substrates [6,7]. The protein is located to the endoplasmic reticulum (ER) but also found in LD at a minor extend. Dga1p needs the presence of K^+ and Mg^{2+} ions for optimal activity [6]. Lro1p, on the other hand, is located exclusively to microsomes (ER) and is the yeast homolog of the human lecithin:cholesterol acyltransferase [8]. It esterifies DG using the acyl-group of phosphatidylethanolamine or phosphatidylcholine as acyl-donor [9]. Lro1p plays a major role in TG synthesis during the exponential phase of growth, whereas Dga1p mediates TG synthesis mainly during the stationary growth phase of the yeast [7]. The two acyl-CoA:cholesterol acyltransferase-related enzymes, Are1p and Are2p, are SE synthases localized to the ER [10,11]. Whereas Are2p prefers ergosterol as a substrate, Are1p uses ergosterol precursors as well. Both Are-proteins catalyze acyl-CoA dependent reactions.

Sandager et al. [12] showed that LD are completely absent in a yeast strain lacking all four non-polar lipid synthesising enzymes, the *dga1Δlro1Δare1Δare2Δ* quadruple mutant (QM). Interestingly, the QM strain is viable and apparent growth defects were not observed under standard conditions. Only when the QM was grown in the presence of exogenous fatty acid, liposensitivity became evident [13].

TG lipases are responsible for TG degradation by cleaving ester bonds of TG [14,15]. Athenstaedt and Daum [15] showed that Tgl3p is the major TG lipase of the yeast *S. cerevisiae*. Tgl3p is mostly located to LD, but was also found in the ER at minor quantities [16]. In a *tgl3Δ* deletion mutant the residual lipase activity is catalyzed by two other lipases named Tgl4p and Tgl5p [14]. In contrast to Tgl3p, which is rather unspecific regarding the chain length of the fatty acids in TG, Tgl4p and Tgl5p exhibit a higher degree of selectivity.

Tgl4p hydrolyzes preferentially TG species with myristic acid (C14:0 acids) and palmitic acid (C16:0), whereas Tgl5p also uses TG with hexacosanoic acid (C26:0) as substrates.
The individual contribution of TG lipases to TG degradation is still a matter of discussion. Tgl3p was shown to be the major TG lipase under standard growth condition [14]. A deletion of *TGL3* resulted in a strongly increased level of TG to ~ 2.7 of wild type, whereas deletion of *TGL4* caused only a moderate change in the total amount of TG. TG lipase activity of Tgl5p was only shown *in vitro*, but deletion of *TGL5* did neither change the overall TG content nor the mobilization of TG *in vivo*. Therefore, it was tempting to speculate about second functions of Tgl4p and Tgl5p. Indeed, our laboratory showed that the major TG lipases do not only act as hydrolytic enzymes, but also contribute to phospholipid synthesis by their function as lysophospholipid acyltransferases. The major TG lipase Tgl3p acylates lysophosphatidylethanolamine in an acyl-CoA dependent manner [17], and Tgl5p exhibits acyl-CoA dependent lysophosphatidic acid acyltransferase activity. Tgl4p, the yeast ortholog of the mouse ATGL (adipocyte triglyceride lipase), is a multifunctional enzyme [18,19]. Besides its TG lipase activity Tgl4p harbours an active center of a phospholipase. Furthermore, Tgl4p acts as a SE hydrolase and an acyl-CoA dependent lysophospholipid acyltransferase.

As described above, TG lipases were studied at the molecular and biochemical level in some detail, but information about regulation of these enzymes is still rare. Recent studies from our laboratory demonstrated that the major TG lipase Tgl3p is strongly regulated by the presence/absence of non-polar lipids [16]. In a yeast strain lacking all non-polar lipid synthesizing enzymes, protein level and stability of Tgl3p were strongly decreased. Furthermore, the protein was located to the ER in the absence of LD. However, both enzymatic activities of Tgl3p, TG lipase and acyltransferase activities, were only attributed to its original localization, the LD.
In the present study we focused on regulatory aspects of the yeast TG lipases Tgl4p and Tgl5p. We mainly addressed the effect of presence/absence of non-polar lipids on the function of these two lipases. Similarities and differences between Tgl3p, Tgl4p and Tgl5p are pointed out.

Materials and Methods

Strains and culture conditions

Yeast strains used in this study are listed in Table 1. Cells were grown aerobically to the stationary growth phase in either YPD media containing 1% yeast extract, 2% glucose and 2% peptone at 30°C; or cultivated in SD medium containing 0.67% yeast nitrogen base (U.S. Biochemical Corp.), 2% glucose and the respective amino acid supplements.

Genetic techniques

Chromosomal tagging and deletions were performed by homologous recombination using the PCR-mediated method described by Longtine et al. [20]. In brief, the inserts for the construction of Tgl4-Myc, Tgl5-Myc, *tgl4*Δ or *tgl5*Δ strains were obtained by PCR from plasmids pFA6a-13Myc-HIS3MX6 or pFA6a-HIS3MX6. Primers used for DNA amplification are listed in Table 2. For transformation of yeast strains, 400-700 ng DNA were used employing the high-efficiency lithium acetate transformation protocol [21]. After transformation, cells were plated on SD medium lacking the respective amino acid. Positive transformants were verified for correct integration of the fusion cassette by analytical PCR of whole yeast cell extracts.

Isolation and characterization of subcellular fractions

LD and microsomal fractions were isolated from cells grown to the stationary phase following published procedures [3,22,23]. The protein concentration of samples was analyzed by the method of Lowry et al. [24] using bovine serum albumin as a standard. Prior to protein determination, LD samples were delipidated with 2-3 volumes of diethyl ether. After withdrawing the organic phase, the residual diethyl ether was removed under a stream of nitrogen. Proteins were precipitated with trichloroacetic acid at a final concentration of 10% and solubilized in 0.1% SDS, 0.1 M NaOH.

SDS-polyacrylamide gel electrophoresis was performed as described by Laemmli [25] using 12.5% separation gels. Western blot analyses was carried out according to Haid and Suissa [26]. Proteins were detected by using rabbit or mouse antisera as the first antibodies and peroxidase-conjugated goat anti-rabbit or anti-mouse as second antibodies. Primary antibodies were directed against the Myc-tag, Wbp1p (ER-marker), GAPDH (cytosolic marker) and Ayr1p (LD marker). Protein amounts of 10-20 µg from each fraction were loaded onto SDS-gels for Western blot analyses. Quantification of immunoreactive bands

detected by Western Blot analyses was performed by measuring the relative intensities of bands using ImageJ program.

Preparation of total cell extract for lipid analysis

Total cell extracts for lipid analyses were prepared from yeast cells grown to the stationary phase and harvested by centrifugation at 3,000 x g for 5 min at room temperature. Cells were suspended in breaking buffer (50 mM TrisHCl, pH 7.4, 150 mM NaCl) with 1mM PMSF. Yeast cells were disintegrated by vigorous shaking in the presence of glass beads for 10 min at 4°C. After cell disruption, cell debris were removed by centrifugation at 3,000 x g for 5 min. The supernatant was used for protein determination and lipid analysis.

Lipid analysis

Lipids from total cell extracts were extracted according to the method of Folch et al. [27] using chloroform/methanol (2:1; v/v) as solvent. For quantification of total phospholipids, lipids from total cell extracts (800 µg protein) were analyzed by the method of Broekhuyse [28]. Individual phospholipids were analyzed from total cell lipid extracts (2 mg protein) by 2-dimensional thin layer chromatography (TLC) using chloroform/methanol/25% ammonia (65:35:5; per vol.) as solvent system for the first dimension; and chloroform/acetone/methanol/acetic acid/water (50:20:10:10:5; per vol.) for the second dimension. Spots were visualized by staining with iodine vapor, scraped off and quantified by the method of Broekhuyse [28].

RNA isolation and Real Time PCR

Total RNA from cells grown to the early-logarithmic phase at 30°C was isolated using the RNeasy kit from Qiagen following the manufacturer's instructions. After DNaseI digestion, Real Time PCR was performed using SuperScript III Platinum SYBR Green One-Step qRT-PCR Kit (Invitrogen) as described by the manufacturer. Reactions were performed in sealed MicroAmp Optical 96-Well Reaction Plates, and amplification was measured using an ABI 7500 instrument (Applied Biosystems). Samples were quantified using the ΔΔCt method described by Livak and Schmittgen [29] with *ACT1* serving as internal control. With this method differences in mRNA expression relative to the control can be calculated. Primers used for Real Time-PCR are listed in Table 3.

Results

Gene expression and protein level of Tgl4p and Tgl5p in cells lacking non-polar lipids

TG lipases Tgl3p, Tgl4p and Tgl5p from *S. cerevisiae* are located to LD where they play an important role in TG mobilization [14,15]. Recently, we showed that protein level, stability and localization of the major TG lipase Tgl3p were regulated by the absence/presence of non-polar lipids [16]. Here, we address the question whether formation of TG and SE also affects properties and function of the two other yeast TG lipases, Tgl4p and Tgl5p.

First, we performed gene expression analyses and determined the protein levels of Tgl4p and Tgl5p in wild type and *dga1Δlro1Δare1Δare2Δ* (QM), which is devoid of non-polar lipids and hence LD. As shown in Figure 1A and 1B, the mRNA expression levels of *TGL4* and *TGL5* were only moderately changed in the QM compared to wild type. This finding was reminiscent of data obtained with *TGL3* [16]. Thus, regulation at the mRNA expression level seems to be irrelevant for all three yeast TG lipases. Next, we tested the protein levels of Tgl4p and Tgl5p in the absence of LD. Interestingly, protein levels of Tgl4p and Tgl5p from total cell extracts were not changed in the *dga1Δlro1Δare1Δare2Δ* mutant compared to wild type (Fig. 1C). This result is in contrast to Tgl3p [16], as the protein level of the major TG lipase was markedly reduced under these condition (Fig. 1C). This was the first major regulatory difference between Tgl3p, Tgl4p and Tgl5p.

Subcellular localization of Tgl4p and Tgl5p in cells lacking LD

In wild type, Tgl4p and Tgl5p are highly enriched in the LD fraction (Fig. 2A, B). This result confirmed previous findings from our laboratory [14]. In this previous study, however, possible dual localizations of Tgl4p and Tgl5p have not been tested. For this reason, other yeast subcellular fractions were also analyzed for the presence of Tgl4p and Tgl4p, respectively. Indeed, significant amounts of Tgl4p and Tgl5p were also found in the ER of wild type. The relative enrichments of Tgl4p and Tgl5p, respectively, in microsomal fractions compared to homogenate are shown in Table 4. As expected, enrichment of the ER marker protein Wbp1p in all microsomal fractions over homogenates was higher than of Tgl4p and Tgl5p. This result can be explained by the dual localization of both TG lipases in LD and the ER. This result is again in strong contrast to Tgl3p which is not enriched in the ER fraction of wild type and rather behaves like a "true" LD protein [16]. The fact that TG lipases Tgl4p and Tgl5p were found at considerable amounts in microsomes was surprising, because the substrate TG is largely missing in the ER. This result raised the question as to the putative function of Tgl4p and Tgl5p in the ER, which will be addressed below.

Considerably amounts of Tgl4p and Tgl5p were also detected in cells lacking LD (see Fig. 1) raising the question as to the localization of these proteins. It has been shown before that several LD proteins including the major TG lipase Tgl3p were retained to the ER when LD were missing [16,30]. Therefore, we speculated that this was also true for Tgl4p and Tgl5p. Figs. 2 D and C and Table 4 show that Tgl4p and Tgl5p were present in microsomal fractions from the QM at similar enrichment as in the wild type. Consequently, there was obviously no shift of Tgl4p and Tgl5p from LD to the ER under depletion of non-polar lipids. This is another marked difference to Tgl3p (see [16]). These data support the idea that Tgl4p and Tgl5p have to be regarded at least in part as true ER proteins.

We also tested whether the absence of their major substrate, TG, caused changes in the subcellular localization of Tgl4p and Tgl5p. It was shown before that the four non-polar lipid synthesizing enzymes Dga1p, Lro1p, Are1p and Are2p contribute differently to LD formation [4]. However, yeast mutant strains lacking either TG or SE are still able to form LD. Preliminary results indicated that Tgl4p and Tgl5p are still localized to LD of a *dga1Δlro1Δ* mutant (Figure 3) indicating that the presence/absence of TG did not affect the subcellular distribution of these enzymes. This result confirmed differences between Tgl3p, Tgl4p and Tgl5p, as localization of the three lipases was not equally affected by the presence of the TG substrate.

Contribution of Tgl4p and Tgl5p to phospholipid synthesis in cells lacking LD

As mentioned before, Tgl3p, Tgl4p and Tgl5p do not only act as TG lipases, but may also contribute to phospholipid synthesis through their lysophospholipid acyltransferase activities [17,19]. Previous results from our laboratory demonstrated, that both enzymatic functions of the major TG lipase Tgl3p were restricted to its original localization, the LD [16]. As Tgl4p and Tgl5p were hardly regulated by non-polar lipid formation and found at substantial amounts in the ER, we speculated that in contrast to Tgl3p, they may play a role in phospholipid synthesis in microsomes. To address this question and to eliminate background effects of Tgl4p and Tgl5p in LD we analyzed total amounts of phospholipids in the QM with an additional deletion of *TGL4* and *TGL5*, respectively, on top. As shown in Figure 4, the total amount of phospholipids was slightly decreased upon deletion of *TGL4* in the QM. This result suggested a moderate, but significant contribution of Tgl4p to phospholipid synthesis when located to the ER. In contrast, a deletion of *TGL5* in the QM did not affect the total cellular amount of phospholipids at all.

To further elucidate the possible role of Tgl4p and Tgl5p in phospholipid synthesis we analyzed individual phospholipids in QM, QM*tgl4*Δ and QM*tgl5*Δ (Table 5). The most significant changes in QM*tgl4*Δ and QM*tgl5*Δ compared to QM were the increased levels of lysophospholipids. Furthermore, a deletion of *TGL4* in the QM led to a decreased level of phosphatidylcholine and an increased level of phosphatidylserine, whereas a deletion of *TGL5* in the QM led to an increased amount of phosphatidic acid. These data supported the view that Tgl4p and Tgl5p are active lysophospholipid acyltransferases in the ER.

Discussion

The present study deals with regulatory aspects of the two yeast TG lipases Tgl4p and Tgl5p caused by depletion of non-polar lipids. In appropriate deletion strain lacking TG or both species of non-polar lipids, TG and SE, we tested biochemical, molecular biological and cell biological properties of Tgl4p and Tgl5p. By using the *dga1*Δ*lro1*Δ*are1*Δ*are2*Δ mutant lacking LD we created a situation, where the two lipases did not have access to the organelle originally identified as their localization.

In a previous study from our laboratory [16] similar questions were addressed with the major yeast TG lipase, Tgl3p. These investigations revealed that Tgl3p when present in a *dga1*Δ*lro1*Δ*are1*Δ*are2*Δ QM was retained in the ER, although at a strongly reduced amount, and became unstable most likely due to inappropriate membrane environment conditions. Results presented here addressing the fate of Tgl4p and Tgl5p under the same conditions yielded different results. These findings led us to reconsider the relationship between the three yeast Tgl-proteins and their possible roles in lipid metabolism.

A clear similarity of Tgl3p, Tgl4p and Tgl5p is the sequences alignment of the three proteins [14,15]. All three enzymes harbor a characteristic lipase motif within a patatin domain, but also an additional enzyme motif characteristic for acyltransferases [17,19]. Tgl3p, Tgl4p and Tgl5p were originally identified as proteins of the LD as shown by proteome analysis and by function [5,14,15]. This view, however, has to be at least in part revised in the light of the findings described in this study and in previous investigations [16]. As demonstrated by careful cell fraction experiments, the three enzymes were not only detected in the LD fraction but also in microsomes (ER). However, clear and important differences were noticed when the subcellular distribution of Tgl3p, Tgl4p and Tgl5p were compared. Whereas the majority of Tgl3p was detected in LD and only smaller amounts in microsomes, the situation with Tgl4p and Tgl5p was different. These two enzymes were found to be less enriched in LD, but

present at amounts in the ER which resembled "true" microsomal proteins (see Fig. 2). This behavior was substantiated in experiments using the *dga1*Δ*lro1*Δ*are1*Δ*are2*Δ quadruple mutant. Whereas Tgl3p retained to the microsomes became unstable and the amount was decreased in the mutant [16], the occurrence of Tgl4p and Tgl5p in the ER of the QM was largely unaffected (see Fig. 2).

Thus, we asked ourselves the question whether or not Tgl3p, Tgl4p and Tgl5p have to be divided into two groups. Tgl3p appears to be a true LD protein, although with dual localization as shown before for several other LD proteins [16,30]. Tgl4p and Tgl5p, on the other hand, seem to behave more like microsomal proteins. We might speculate that the amounts of these two proteins in LD are in a parking lot. This hypothesis, of course, raises the question as to the function of the three enzymes in the living cell. Whereas Tgl3p, which exhibits the major TG lipase activity *in vivo* and *in vitro*, appears to be concentrated near its substrate, the TG of LD, Tgl4p and Tgl5p seem to be present at least at large amounts separated from the TG substrate. Thus, we are left with a possible role of the three Tgl-proteins in the ER. Also in this case, there seems to be a clear difference between Tgl3p, Tgl4p and Tgl5p. Whereas Tgl3p was found to be practically inactive in microsomes [16], Tgl4p and Tgl5p present in microsomes were shown to affect the phospholipid pattern of the cell (see Fig. 4 and Table 5). The slight depletion of total phospholipids in a *dga1*Δ*lro1*Δ*are1*Δ*are2*Δ*tgl4*Δ indicated that the function of Tgl4p as a lysophospholipid acyltransferase, but also as a phospholipase may be envisaged. As the enzyme is only present in the ER in this mutant strain, both enzymatic activities have to be attributed to the microsomal portion of Tgl4p. Tgl5p seems to change total amounts of major lipid groups in the cell less actively. Our previous studies have shown that the total amount of TG was not changed in a *tgl5*Δ mutant [14] although internal changes in the species distribution of TG were observed. Thus, Tgl5p was rather regarded as a TG remodeling enzyme than a lipase. This view may be supported by our findings shown here (see Fig. 4 and Table 5). The total amount of phospholipids in a *dga1*Δ*lro1*Δ*are1*Δ*are2*Δ*tgl5*Δ mutant was similar to the QM, but the pattern of individual phospholipids was slightly changed. Accumulation of lysophospholipids in a pentuple mutant strain may serve as an argument for Tgl5p as a lysophospholipid acyltransferase when present in the ER. As for Tgl4p, the activity of Tgl5p in microsomes confirmed the difference to Tgl3p.

Taken together, results presented in this study broaden our knowledge of the metabolic roles of Tgl-proteins in the yeast. Although originally being identified as TG lipases, the function of these enzymes in lipid metabolism may be broader on one hand, but probably more

specific on the other hand. Thus, different regulatory aspects applying to Tgl3p, Tgl4p and Tgl5p have also to be taken into account. The experimental set-up employed in this work may be regarded as an appropriate strategy and a first step to address these questions and to shed more light on the function(s) of these proteins *in vivo*.

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Abbreviations: The abbreviations used are: ER, endoplasmic reticulum; LD, lipid droplets; QM, quadruple mutant; SE, steryl esters; TG, triacylglycerols; TLC, thin layer chromatography.

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Tables

Table 1 Yeast strains used in this study

Strain	Genotype	Source
Wild type	BY4741 *Mat a; his3Δ1; leu2Δ0; met15Δ0; ura3Δ0*	Euroscarf
QM	BY4741; *dga1*Δ::*kanMX4 lro1*Δ::*kanMX4 are1*Δ::*kanMX4 are2*Δ::*kanMX4*	[31]
QM*tgl4*Δ	QM; *tgl4*Δ::*HIS3MX6*	This study
QM*tgl5*Δ	QM; *tgl5*Δ::*HIS3MX6*	This study
Tgl4-Myc	BY4741; *TGL4-13Myc*::*HIS3MX6*	This study
QM Tgl4-Myc	QM; *TGL4-13Myc*::*HIS3MX6*	This study
Tgl5-Myc	BY4741; *TGL5-13Myc*::*HIS3MX6*	This study
QM Tgl5-Myc	QM; *TGL5-13Myc*::*HIS3MX6*	This study
*dga1*Δ*lro1*Δ Tgl4-Myc	BY4741; *lro1*Δ::*URA3KL dga1*Δ::*kanMX4 TGL4-13Myc*::*HIS3MX6*	This study
*dga1*Δ*lro1*Δ Tgl5-Myc	BY4741; *lro1*Δ::*URA3KL dga1*Δ::*kanMX4 TGL5-13Myc*::*HIS3MX6*	This study

Table 2 Primers used throughout this study

Primer	Sequence (5'→3')
Tgl4F2	CGAGGCCTTCTTCTTCAACGCAGCACAAAAGCACCACCAGTTTTACTCAACGGATCCCCGGGTTAATTAA
Tgl4S2	CATAGATGAAAAAGAATATCTAGAGGATATATAAGCAAGCCCGTGTTTTCATCGATGAATTCGAGCTCG
Tgl4S1	TGTAATAATTATTGAAGGGAGTACAGGTATATGTAATAAAAGTCTGAATGCGTACGCTGCAGGTCGAC
Tgl5F2	CGGCTGCCACAAACGACAATTTCATGAACAATTCAGACATTTTTCAAAATCGGATCCCCGGGTTAATTAA
Tgl5S2	TGAGAATATAGAAAGCTTTTTATATAAAAATGTACTTATTGTCTTTCATTTCAATCGATGAATTCGAGCTCG
Tgl5S1	AAAAGACATCATAAACAGCACAAGGAAGACGGTTCTGTTTCGTTGCTATGCGTACGCTGCAGGTCGAC

Table 3 Primers used for RT-PCR

Primer	Sequence (5'→3')
RT-Act1-fwd	CCAGCCTTCTACGTTTCCATCCAAG
RT-Act1-rev	GACGTGAGTAACACCATCACCGGA
RT-Tgl4-fwd	TGCCCGACATGTGTATGCTTTTTAGAAT
RT-Tgl4-rev	CTTGGGCCACGTAGCTTTTGCAC
RT-Tgl5-fwd	CCGGGAGTTGACTTGGAAGAATCC
RT-Tgl5-rev	GGAGAAGGCAATGGCTGAAGAGGA

Table 4 Relative enrichment of Tgl4p and Tgl5p in different subcellular fractions from the wild type (WT) and *dga1*Δ*lro1*Δ*are1*Δ*are2*Δ (QM) strain. Relative enrichments of Tgl4p-Myc or Tgl5p-Myc, ER-marker protein Wbp1p and cytosolic marker protein GAPDH in homogenate, 30,000 x g microsomes and 40,000 x g microsomes are shown. Relative enrichment factors were calculated using ImageJ with mean deviation of ±10%. The relative enrichment of proteins in homogenate was set at 1.

	WT				QM			
	Tgl4p	Tgl5p	Wbp1p	GAPDH	Tgl4p	Tgl5p	Wbp1p	GAPDH
Homogenate	1	1	1	1	1	1	1	1
Microsomes 30,000xg	2	1.8	~4.5	0.2	2.5	3	~6.5	n.d
Microsomes 40,000xg	1.9	1.2	~5.5	0.1	1.7	2.1	~4.5	n.d

Table 5 Phospholipid composition of cell free homogenate from cells grown on YPD. QM, quadruple mutant; LPL, lysophospholipids; PI, phosphatidylinositol, PS, phosphatidylserine; PE, phosphatidylethanolamine; PC, phosphatidylcholine; DMPE, dimethylphosphatidylethanolamine; CL, cardiolipin; PA, phosphatidic acid. Mean values of at least three measurements with standard errors are shown.

Strain	Phospholipids in cell free homogenate (mol %)							
	LPL	PI	PS	PC	PE	DMPE	CL	PA
QM	0.2±0.2	8.9±1	3.0±0.9	55.3±6	24.2±3.8	6.0±0.9	0.3±0.2	2.1±0.4
QM*tgl4*Δ	1.7±1.04	12.5±1.8	4.6±0.9	47.7±6.9	23.4±1.8	7.4±2.1	0.6±0.5	2.7±0.6
QM*tgl5*Δ	2.2±1	10.0±1.5	2.7±0.2	52.0±4.8	21.6±2.2	6.5±0.5	0.6±0.2	4.3±0.4

FIGURE 1

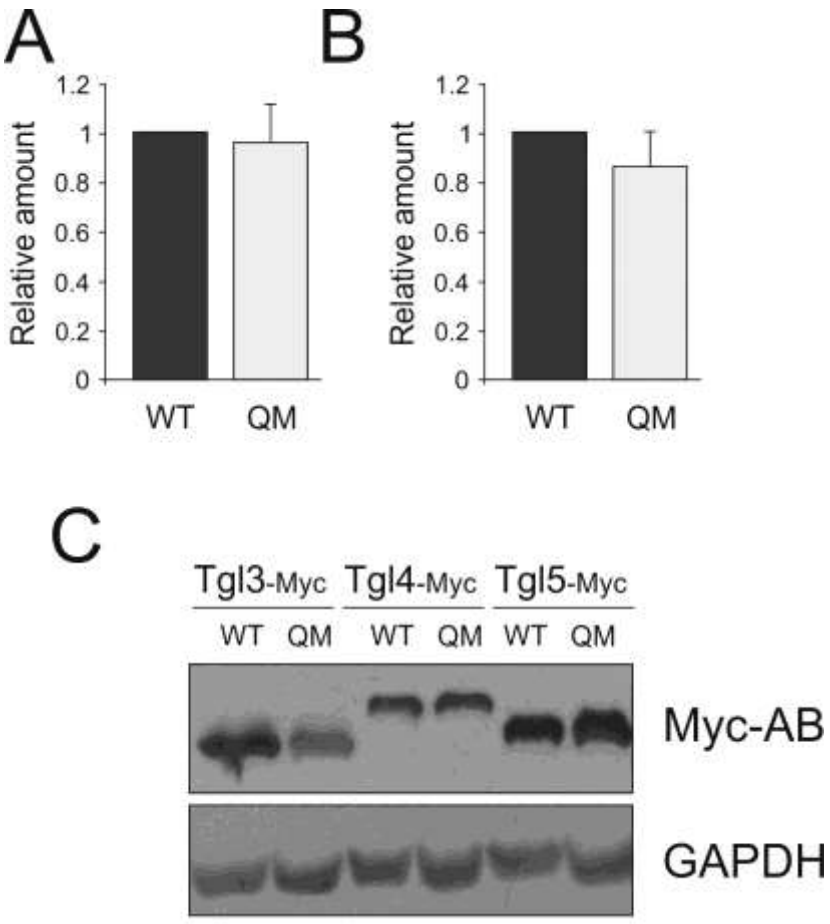

Fig. 1 Gene expression and protein level of Tgl4p and Tgl5p in the absence of lipid droplets. Relative gene expression of (A) *TGL4* and (B) *TGL5* in wild type (WT) (black bar) and *dga1*Δ*lro1*Δ*are1*Δ*are2*Δ QM (grey bar) was measured by RT-PCR. Wild type was set at 1. Data are mean values from three independent experiments with the respective deviation. (C) Protein analysis of Tgl3-Myc, Tgl4-Myc and Tgl5-Myc from total cell extracts of wild type and QM grown to the stationary phase. The primary antibody was directed against the Myc-tag (Myc-AB). The cytosolic marker GAPDH was used as loading control. Western blot analyses are representative of at least two independent experiments.

FIGURE 2

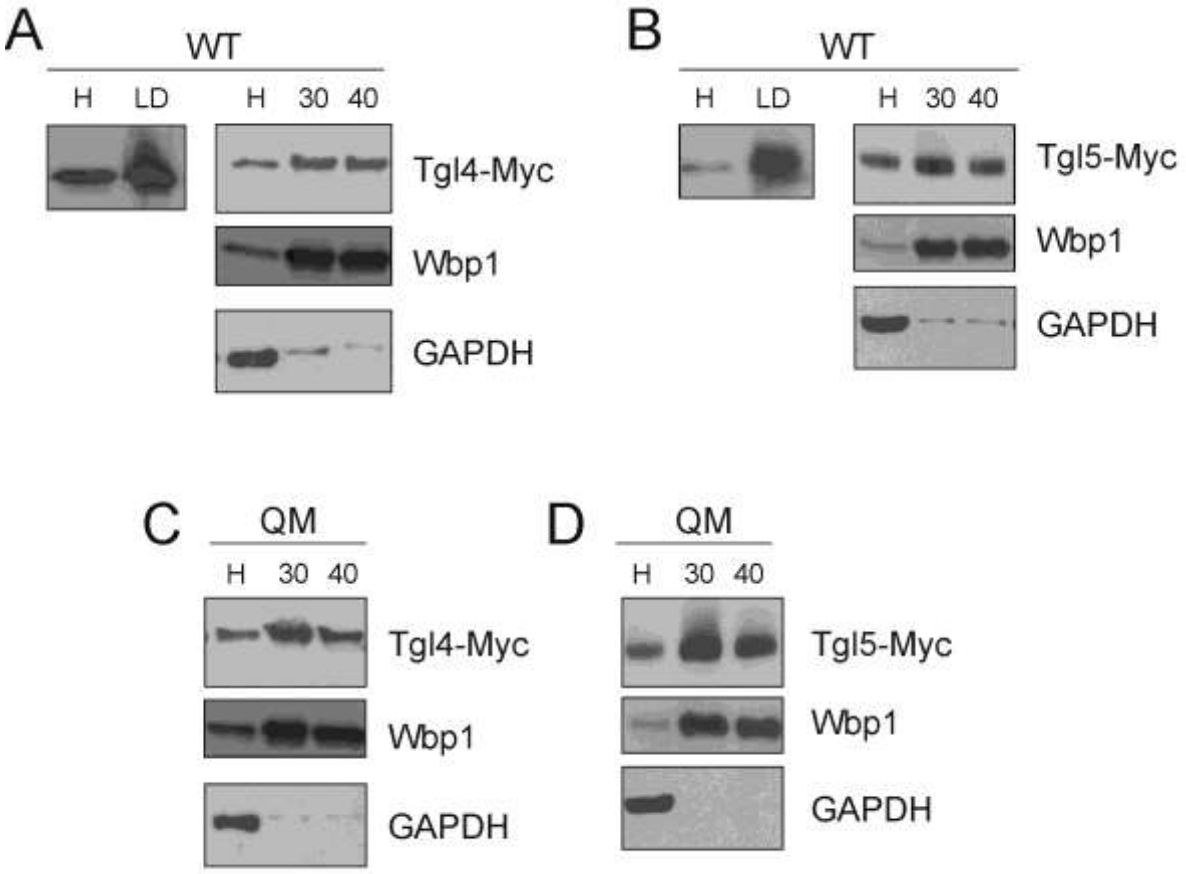

Fig. 2 Localization of Tgl4p and Tgl5p in the wild type (WT) and *dga1*Δ*lro1*Δ*are1*Δ*are2*Δ (QM) strain. Western blot analysis of Tgl4-Myc and Tgl5-Myc in the homogenate (H), 30,000 x g microsomal (30), 40,000 x g microsomal (40) and LD fractions from WT (A, B) and the QM (C, D), respectively, grown to the stationary phase. Primary antibodies were directed against the Myc-tag, Wbp1p (ER-marker) and GAPDH (cytosolic marker). Western blot analyses are representative of at least two independent experiments.

FIGURE 3

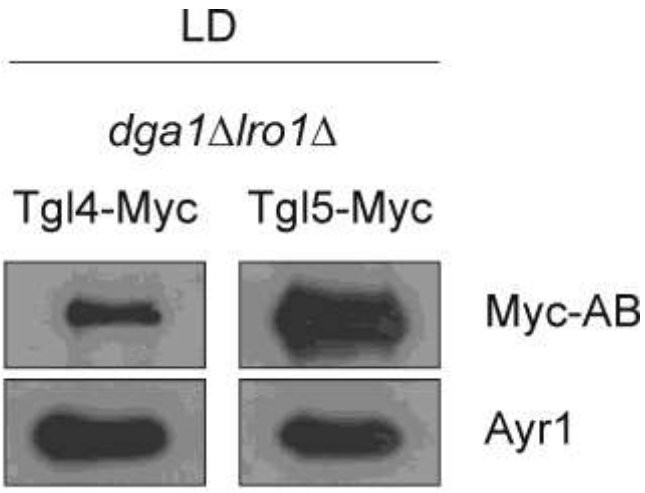

Fig. 3 Localization of Tgl4p and Tgl5p in yeast strains lacking triacylglycerol synthases. Western blot analysis of Tgl4-Myc and Tgl5-Myc in LD fractions from *dga1*Δ*lro1*Δ grown to the stationary phase. Primary antibodies were directed against the Myc-tag (Myc-AB) and Ayr1p (LD marker). Western blot analyses are representative of at least two independent experiments.

FIGURE 4

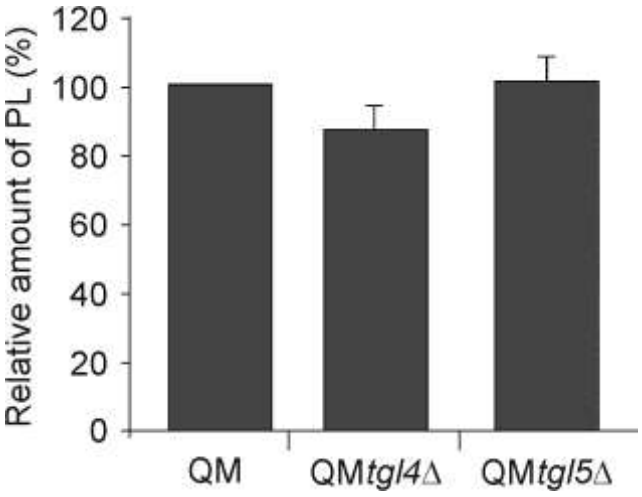

Fig. 4 Phospholipid analysis of *dga1*Δ*lro1*Δ*are1*Δ*are2*Δ (QM) strains lacking triacylglycerol lipases Tgl4p and Tgl5p, respectively. Relative amounts of total phospholipids/mg protein from total cell extracts of QM, QM*tgl4*Δ and QM*tgl5*Δ grown to the stationary phase are shown. Data are mean values of two independent experiments performed in duplicate with the respective deviations. Wild type was set at 100%.

Summary and General Discussion

Lipids are important compounds of biological membranes, which are constituents of internal compartments. For the formation of cellular membrane lipids, fatty acids need to be available during cell growth. Therefore, fatty acids are stored in biological inert forms of non-polar lipids triacylglycerols (TG) and steryl esters (SE), (for review see Czabany et al. 2007; Rajakumari et al. 2008; Daum et al. 2007). Four enzymes contribute to non-polar lipid biosynthesis in the yeast, namely the two TG synthases Dga1p and Lro1p, and the SE synthases Are1p and Are2p (see Fig. 1). TG and SE can be mobilized upon requirement, e.g. under substrate limitation, by the TG lipases Tgl3p, Tgl4p and Tgl5p and by the SE hydrolases Tgl1p, Yeh1p and Yeh2p, respectively. Catabolic as well as anabolic pathways of non-polar lipid metabolism are well described in the yeast *S. cerevisiae*, but data about molecular regulatory mechanisms concerning non-polar lipid storage and mobilization are still rare. This study provides some evidence to understand the complex network of non-polar lipid metabolism in the yeast in more detail.

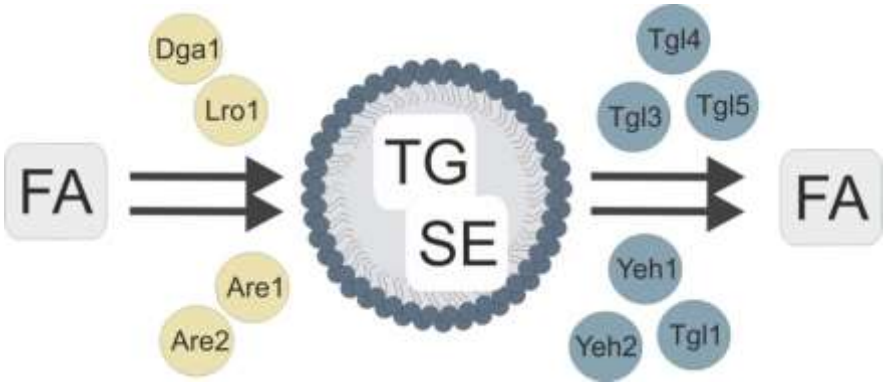

Fig. 1 Non-polar lipid metabolism in the yeast.

The three patatin domain-containing TG lipases Tgl3p, Tgl4p and Tgl5p and their contribution of TG hydrolysis has been characterized previously in our laboratory (Athenstaedt & Daum 2005, 2003). The fact, that these enzymes do not only have one hydrolytic activity but two enzymatic functions (lipase and acyltransferase) opened a novel view on these proteins with respect to their function and regulation (Rajakumari & Daum 2010b, 2010a; Rajakumari et al. 2010). Previous results from our laboratory revealed that in cells entering the vegatitve growth phase from the stationary phase lipolysis is induced (Athenstaedt & Daum 2005). Kurat et al. (2009) demonstrated, that Tgl4p, which is the functional ortholog of murine adipocyte TG lipase (ATGL), activates lipolysis and early bud

formation in the late G1 phase of the cell cycle upon phosphorylation by cyclin-dependent kinase 1 Cdk1p/Cdc28p. Lack of phosphorylation leads to delayed bud formation and cell-cycle progression. Ptacek et al. (2005) showed by global analysis of protein phosphorylation that Tgl3p is a target of non-essential kinase Pho85p. However, details about how TG lipases coordinate and balance formation, storage and mobilization of TG are still a matter of discussion. Results demonstrated here give first insight into regulation of yeast TG lipases. We showed that regulation of TG lipases depends on the absence/presence of non-polar lipids. In the absence of lipid droplets (LD) or sufficient substrate, the protein level and protein stability of Tgl3p are strongly reduced. Additionally, Tgl3p is retained in the ER under these conditions. Because Tgl3p lacks lipolytic as well as acyltransferase activity when located to the ER, we conlcuded that the ER serves as a kind of parking compartement for Tgl3p in the absence of LD and sufficient substrate.

Another open question is the contribution of single TG lipases to TG hydrolysis in the living cell. Results from our laboratory showed that the three TG lipases contribute differently to TG turnover (Athenstaedt & Daum 2005). Interestingly, in a yeast strain devoid of the major TG lipases, TG hydrolysis does not completely come to an halt, indicating the presence of additional TG lipases. Indeed, Ploier et al. (2013) identified Ayr1p as a novel not yet characterized TG lipase. However, also a *tgl3*Δ*tgl4*Δ*tgl5*Δ*ayr1*Δ deletion strain showed residual TG lipolytic activty suggesting the existance of further lipases and hydrolases with lower activities. Ploier et al. (2013) demonstrated that the action of such enzymes may depend on growth conditions, e.g. on the carbon source.

Tgl3p, Tgl4p and Tgl5p do not only act as TG lipases but also contribute to phospholipid synthesis as lysophospholipid acyltransferases (Rajakumari & Daum 2010b, 2010a; Rajakumari et al. 2010). It was demonstrated, that these activities do not occur at the same active center. Thus, TG lipases contain independent reactive domains for lipolysis and acyltransferase reaction, respectively. However, lipolysis and re-esterification by TG lipases might be tighly linked processes. How TG lipases balance lipolytic and synthetic activities upon requirement still needs to be addressed. The biosynthetic activities of the enzymes as lysophospholipid acyltransferases might be used for LD growth by providing phospholipids for the formation of the surface monolayer membrane. On the other hand, Tgl3p, Tgl4p and Tgl5p may provide phospholipids for different organelles, e.g. the ER. Results by Rajakumari and Daum (2010a) already suggested that TG lipases are directly involved in membrane lipid biosynthesis during sporulation, as sporulation efficiency was strongly reduced in a *tgl3*Δ

strain. Additionally, however fatty acids and/or complex lipids formed by the action of the three Tgl enzymes in LD may be provided for other organelles, e.g. the ER. A close relationship of LD to other organelles was discussed. Jacquier et al. (2011) demonstrated that LD are functionally connected to the ER in *S. cerevisiae*. Association of LD with the ER seemed to be either permanent or to occur frequently enough to transfer proteins between the surfaces of the two organelles. Therefore, LD are regarded as to be dynamic and interactive organelles. Moreover, LD are often found in close proximity to mitochondria, lysosomes or peroxisomes (for review see Kohlwein et al. 2013). Membrane association between LD and different organelles is probably a prerequisite for lipid transport between the organelles, and therefore membrane contact sides might exist for lipid exchange. A similar contact site, the so-called MAM (Mitochondria Associated ER Membrane fraction), was identified between mitochondria and the ER (Ardail et al. 1993; Achleitner et al. 1999).

TG form the hydrophobic core of LD surrounded by several outer shells of SE and a phospholipid monolayer (Czabany et al. 2008). This extraordinary structure raised the question about the topology of polypeptides when located to LD. Similar to other LD proteins, TG lipases lack classical transmembrane spanning domains and are therefore assumed to reside in the phospholipid monolayer of the organelle. However, this topology raises the question how lipases get access to their substrates. Experimental evidence suggested that the C-terminus of various LD proteins is very important for protein stability. Müllner et al. (2004) showed that lack of the hydrophobic C-terminus of Erg1p, Erg6p and Erg7p, proteins involved in ergosterol biosynthesis, prevented the association of the proteins with LD and caused their retention to the ER. More recently, Koch et al. (2014, manuscript under revision) demonstrated that the C-terminus of Tgl3p faces the inside of the LD or is at least protected by the phospholipid monolayer of the organelle. In contrast, the N-terminus of Tgl3p faces the cytosolic side of the LD. Information about the orientation of active centres of LD enzymes is still missing. Further topology studies with TG lipases will address this problem.

In this thesis it was demonstrated that the functionality of the major TG lipase Tgl3p may indeed depend on its topology. When inserted into the surface phospholipid monolayer of LD, Tgl3p plays an important role in TG and phospholipid metabolism. In contrast, the portion of Tgl3p inserted into a phospholipid bilayer of the ER membrane did contribute to lipid metabolism. Results by Koch et al. (2014, manuscript under revision) have already indicated that the topology of Tgl3p changes when the protein moves from the ER to LD. We can

speculate that this change in topology has an impact on the activity of Tgl3p. Interestingly, it appears that Tgl4p and Tgl5p can cope much better with different membrane environments than Tgl3p. In contrast to Tgl3p, Tgl4p and Tgl5p seem to contribute to phospholipid metabolism as lysophospholipid acyltransferases when located to the ER. Their lipolytic activities, however, seem to be restricted to the LD.

In the yeast *S. cerevisiae*, the four enzymes Dga1p, Lro1p, Are1p and Are2p contribute differently to non-polar lipid synthesis (Oelkers et al. 2002; Sandager et al. 2002; Sorger & Daum 2002; Dahlqvist et al. 2000). In this Thesis a regulatory link between non-polar lipid synthesis and degradation in the yeast was shown. A block in TG hydrolysis led to altered non-polar lipid synthesis and accumulation of TG upon block of TG hydrolysis caused feedback regulation to the synthesis of TG and SE. Whereas the formation of TG was strongly reduced, the synthesis of SE was enhanced. Interestingly, fatty acid biosynthesis was not reduced under such conditions. Thus, it was concluded that the enhanced formation of SE is a way out to avoid fatty acid accumulation. However, the molecular mechanism of this metabolic shift is still unknown. Neither gene expression nor protein levels of proteins involved were changed. Therefore, we assume that regulation of the activities occurs on the enzymatic level. Positive and/or negative regulators of non-polar lipid synthesizing enzymes may be ivolved, but were not yet identified.
Attempts to understand molecular and cellular regulation of non-polar lipid synthesis were already made in the past. As an example, regulation of SE synthases *ARE1* and *ARE2* was shown to be oxygen-dependent (Valachovic et al. 2001). Kamisaka et al. (2007) demonstrated that overexpression of *DGA1* in a strain deleted of *SNF2*, a transcription factor which is part of the SWI/SNF chromatin remodelling complex, caused enhanced diacylglycerol acyltransferase activity. The enhanced enzymatic activity in this strain was not due to an increased amount of Dga1p (Kamisaka et al. 2007, 2006). These experiments led to the speculation, that Dga1p may be activated by post-translational modifications. Indeed, Dga1p and Are2p were identified as a target for protein kinases (Ptacek et al. 2005). However, these findings were based on a global phosphorylation screening and need further evaluation.

Regulatory links between non-polar lipid and phospholipid metabolism have also been investigated. Horvath et al. (2011) demonstrated a metabolic link between phosphatidylethanolamine and TG metabolism in the yeast. TG synthase 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) pathway. Interestingly, the transcription level of *LRO1* was not affected. The authors speculated, that the insufficient supply of phosphatidylethanolamine as substrate for phospholipid:diacylglycerol acyltransferase caused the strongly reduced Lro1p activity and the decreased TG level. It was concluded that TG and phosphatidylethanolamine synthesis are tightly linked in the yeast. Gaspar et al. (2011) reported a cross-talk between TG metabolism and phosphatidylinositol synthesis. These authors showed that the *tgl3Δtgl4Δtgl5Δ* strain, which is largely unable to mobilize TG, also exhibited attenuated phosphatidylinositol formation. Furthermore, Malanovic et al. (2008) showed that phosphatidylcholine *de novo* synthesis and TG synthesis were metabolically linked processes through the phospholipid methylation reaction, since down-regulation of *SAH1*, the *S*-adenosyl-l-homocysteine hydrolase, caused decreased *de novo* synthesis of phosphatidylcholine, increased TG formation and LD proliferation. Another link between non-polar lipid and phospholipid metabolism was shown in this PhD thesis. Accumulation of TG and changes in non-polar lipid formation also affected phospholipid formation, at least to some extent. Interestingly, the increased formation of phospholipids in cells lacking TG lipases caused only a moderate change in the total amount of phospholipids. This result may be regarded as a first hint for additional regulatory mechanisms maintaining balanced phospholipid levels in the cell.

In conclusion, this Thesis led to a better understanding of regulatory links within the lipid metabolic pathways. Not surprisingly, however, these studies demonstrated that we are just beginning to understand this complex regulatory network. The model system yeast will hopefully be employed to study such aspects also in future investigations, and thus contribute to our general understanding of lipid biology.

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Curriculum Vitae

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EDUCATION

Feb.2011- dato	**PhD study Biotechnology, Biochemistry and Food Chemistry** *Graz University of Technology, Austria* Thesis: Triacylglycerol lipases of the yeast
Aug.2013	**PhD Summer school** Biological Mass Spectrometry Faculty of Mathematics and Science *University of Jyväskylä, Finland*
Oct.2008- Jan.2011	**Masters study Molecular Microbiology** *Karl-Franzens-University Graz, Austria* Degree: Master of Science, passed with distinction Thesis: Investigation of late cytoplasmic pre-60S maturation steps
Oct.2005- Jun.2008	**Bachelors study Molecular Biology** *Karl-Franzens-University Graz, Austria* Degree: Bakk. rer. nat.
Sep.2001- Jun.2005	Technologierealgymnasium Kirchdorf/Krems, Österreich Degree: **Matura,** passed with distinction

WORKING EXPERIENCE

Feb.2011- dato **Institute of Biochemistry**

Graz University of Technology, Austria

Project Assistant, Focus on:

- Protein analytics: Enzyme Assays, Immunoassays
- Lipid analytics: Extraction, GLC, TLC
- Construction and engineering of yeast strains

Teaching Assistant for the lab course Immunological Methods (4 ECTS)

Supervision of Master- and Project students

Nov.2009- Jan.2011 **Institute of Molecular Biosciences**

Karl-Franzens-University Graz, Austria

Student Assistant, Focus on:

- Protein analytics: Recombinant protein expression in *E. coli* and yeast, protein purification with affinity chromatography, protein determination, SDS-PAGE, Western Blot
- DNA/RNA analytics: Cloning, sequencing, qRT-PCR, electrophoresis, genetic interaction studies

Tutor for the laboratory courses of Microbiology (4 ECTS), Yeast Cell Biology (6 ECTS) and Advanced Yeast Cell Biology (8 ECTS)

2003-2010 **Laboratory, Quality Assurance, Production/Packaging**

Piesslinger GmbH, Molln, Austria

Summer job of a total of 12 months, working in the Quality Assurance on quality tests of ELOXAL and powder-covered aluminum;

Working in shift operation at the production/packaging site ELOXAL

Sep.2008/Jul.2009 **Laboratory of Molecular Biology and Tumorcytogenetic**

Hospital of the Sisters of Mercy Linz, Austria

Summer job, DNA/RNA preparation of blood and tissue cells, working in an aseptic environment; Insights into FISH, cell cultures, karyograms

Jul./Aug.2008 **Genomics Research Group**

Transfusion Centre Linz, Austria

Internship, Validation of Bacteria-Screenings, Sequencing; Insights into Genome Sequencing Method LifeScience454®

ADDITIONAL EXPERIENCES

Jun.2013 **Academic Seminar** Industrial Biotechnology (3-days)
Sandoz GmbH, Kundl, Austria

SKILLS and ACHIEVEMENTS

Languages: German (native)
English (fluent)
Spanish; Latin (basics)

IT- Skills: Microsoft Office, SerialCloner, Bioinformatic Tools, CoralDraw, Coral PhotoPaint, AdobePhotoshop

Additional Skills: Driving License B

Awards & Grants: PYFF5 Student Fellowship 2013
FEBS Youth Travel Grant 2012

Interests & Activities: Member of the Austrian Association of Molecular Life Sciences and Biotechnology (ÖGMBT)
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Publications

1. Koch B.#, Schmidt C.# and Daum G. Storage Lipids of Yeast. (2014) *FEMS Microbiol Rev.* [manuscript accepted] # Equal contribution
2. Loibl M., Klein I., Prattes M., Schmidt C., Kappel L., Zisser G., Gungl A., Krieger E., Pertschy B. and Bergler H. The Drug Diazaborine Blocks Ribosome Biogenesis by Inhibiting the AAA-ATPase Drg1. (2013) *J Biol Chem.* 289:3913-22
3. Ploier B., Scharwey M., Koch B., Schmidt C., Schatte J., Rechberger G., Kollroser M., Hermetter A. and Daum G. Screening for hydrolytic enzymes revealed Ayr1p as a novel triacylglycerol lipase in *Saccharomyces cerevisiae*. (2013) *J Biol Chem.* 288(50):36061-72
4. Schmidt C.#, Ploier B#., Koch B. and Daum G. Analysis of Yeast Lipid Droplet Proteome and Lipidome. (2013) *Methods Cell Biol.* 116:15-37 # Equal contribution
5. Schmidt C., Athenstaedt K., Koch B., Ploier B. and Daum G. Regulation of Triacylglycerol lipase Tgl3p by formation of non-polar lipids. (2013) *J Biol Chem.* 288(27): 9939-48
6. Baßler J., Klein I., Schmidt C., Kallas M., Thomson E., Wagner M.A., Bradatsch B., Rechberger G., Strohmaier H., Hurt E. and Bergler H. The conserved Bud20 zinc finger protein is a new component of the ribosomal 60S subunit export machinery. (2012) *Mol Cell Biol.* 32(24):4898-912

Oral presentation

1. Schmidt C., Athenstaedt K., Koch B., Ploier B. and Daum G. Regulation of Triacylglycerol lipase Tgl3p by formation of non-polar lipids. 5th Conference on Physiology of Yeast and Filamentous Fungi, Montpellier, France, June 4th-7th 2013

Poster presentations

1. Schmidt C., Athenstaedt K. and Daum G. Regulation of triacylglycerol lipase Tgl3p by non-polar lipids. Keystone Symposia Lipids in Biology and Disease, Dublin, Irland, March 19th-24th 2012
2. Schmidt C., Athenstaedt K. and Daum G. Regulatory link between non-polar lipid synthesis and degradation in the yeast *Saccharomyces cerevisiae.* Joint Annual Meeting of ÖGMBT, Graz, Austria, September 17th-20th 2012
3. Schmidt C., Athenstaedt K. and Daum G. Regulatory link between non-polar lipid synthesis and degradation in the yeast *Saccharomyces cerevisiae.* 2nd European Symposium on Microbial Lipids, Bern, Switzerland, May 16th -19th 2012