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Regulation of steryl ester metabolism in the yeast *Saccharomyces cerevisiae*

DISSERTATION

zur Erlangung des akademischen Grades

Doktor der Naturwissenschaften

eingereicht an der

Technischen Universität Graz

Betreuer

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

Institut für Biochemie

Technische Universität Graz

Graz, Oktober 2017

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Danksagung

Aufrichtiger Dank gebührt meinem Betreuer Prof. Günther Daum, der mich nicht nur während meiner Dissertation sondern die vergangenen fünf Jahre hindurch stets begleitet und geführt hat. Neben seiner vollsten Hingebung für die Forschung war er sich stets der Verantwortung seinen Studenten und Angestellten gegenüber bewusst. Die zahlreichen konstruktiven Gespräche auf intellektueller sowie auch persönlicher Ebene werden mir immer als bereichernder Austausch in Erinnerung bleiben.

Einen ganz besonderen Dank richte ich an Karin Athenstaedt. Durch ihre mannigfache Ideengebung war sie mir während all der Jahre Quell der Inspiration und schaffte es, mir geistige Anregungen zu geben. Dankbar anerkennen will ich auch Ihre immer freundliche, uneingeschränkte und geduldige Bereitschaft mir eine Stütze zu sein, wann immer es notwendig war.

Wichtig für meinen universitären Werdegang und Fortschritt waren neben Karin und Günther die beiden weiteren Mitglieder meines Thesis Committees, Herr Prof. Karl Lohner und Herr Prof. Kai-Uwe Fröhlich. Danke für die wissenschaftlichen Ratschläge und das angenehme Zusammenarbeiten. Danke auch den finanziellen Förderern wie dem Doktoratskolleg Molecular Enzymology, dem FWF, EMBO und FEBS.

Herzlichen Dank richte ich an dieser Stelle auch an Prof. Roger Schneiter. Er hat mich in den sechs Monaten meines Auslandsaufenthaltes in Fribourg mit seinem unerschöpflichen Fundus an thematischen wissenschaftlichen Hinweisen stets in neue Sphären gelenkt.

Enorm wichtig für ein gelungens Ganzes ist das persönliche Wohlbefinden in einem gemeinschaftlichen Miteinander, begleitet von Toleranz, Wertschätzung und Respekt. Vielen lieben Dank an die „Daumies“, die das freundliche Zusammensein ganz maßgebend geprägt haben. Danke den Institutsmitgliedern, die dieses harmonische Klima gefördert haben. Ganz lieben Dank dem Schneiter lab für die unheimlich liebevolle und tolle Integration in die Gruppe und die vielen wunderbaren Momente.

Meinen Freunden und meiner Familie ein ganz großes Dankeschön. Ihr habt meine Pläne stets bestärkt und mir den Rücken frei gehalten. Besonders wertvoll war für mich auch die offene und ehrliche Kritik, die mich, immer wenn es nötig war, geerdet hat. Tausend Dank meiner Mama und meinen Schwestern, Ihr seid einfach spitze. Ich bin froh und äußerst glücklich, dass ich Euch habe.

Zusammenfassung

Die zwei wichtigsten Speicherlipide in der Hefe *Saccharomyces cerevisiae* sind die Neutrallipide Triglyceride und Sterolester. Beide werden im Endoplasmatischen Reticulum synthetisiert. Die Enzyme Dga1p und Lro1p sind für die Bildung von Triglyceriden zuständig. Are1p und Are2p dienen hauptsächlich der Synthese von den Sterolestern, tragen allerdings auch, wenngleich zu einem sehr geringen Anteil, zur Triglyceridsynthese bei. Sterolester und Triglyceride werden in organell-ähnlichen Strukturen, den Lipidpartikeln gespeichert und können während des Wachstums oder in Hungerphasen von hydrolysierenden Enzymen mobilisiert werden, um ausreichend Energie und Membrankomponenten zur Verfügung zu stellen. Die Triglyceride werden von Triglyceridlipasen gespalten, und die Sterolester von den drei Sterolesterhydrolasen Tgl1p, Yeh1p and Yeh2p. Während Tgl1p und Yeh1p an den Lipidpartikeln lokalisiert sind, ist Yeh2p eine Sterolesterhydrolase an der Zellperipherie. Das Hauptthema meiner Doktorarbeitet war es die regulatorischen Aspekte des Sterolester-metabolismus zu studieren. Einerseits wollten wir wissen, wie die zwei Sterolestersynthasen auf ein Fehlen der Sterolester spaltenden Enzyme reagieren. Wir konnten zeigen, dass Are1p und Are2p im Bezug auf die drei Sterolesterhydrolasen feedback reguliert sind. Zwar gab es keine Veränderungen der Genexpression und des Proteingehalts der zwei Acyltransferasen bei Nichtvorhandensein ihrer Gegenspieler, sehr wohl aber waren die *in vitro* Aktivität von Are1p und Are2p und ihr Potential *in vivo* radioaktiv markierte Fettsäuren in Sterolester einzubauen signifikant eingeschränkt. Andererseits analysierten wir das Verhalten der drei Sterolesterhydrolasen in Gegenwart oder Abwesenheit von Neutrallipiden. Dazu verwendeten wir Stämme denen entweder Sterolester, Triglyceride oder sogar die Lipidpartikel per se fehlten. Nur wenn die Zellen keine Lipidpartikel mehr aufbauen konnten, wurden Tgl1p und Yeh1p am Endoplasmatischen Retikulum retiniert. Dort zeigten sich die Enzyme äußerst instabil und verloren ihre Fähigkeit, Sterolester zu spalten. Weiters konnten wir bestätigen, dass Yeh2p an der Plasmamembran lokalisiert und auch an der Plasmamembran bleibt, auch wenn die Zellen keine Lipidpartikel mehr besitzen, und ein phosphoryliertes Protein ist. Zusammenfassend tragen die Ergebnisse dieser Doktorarbeit zum Verständnis einiger regulatorischer Aspekte des Sterolestermetabolismus in der Hefe bei.

Abstract

The two major storage lipids in the yeast *Saccharomyces cerevisiae* are the nonpolar lipids triacylglycerol and steryl esters. Both lipid species are synthesized in the endoplasmic reticulum. Dga1p and Lro1p are responsible for the formation of triacylglycerols and Are1p and Are2p catalyze the synthesis of steryl esters. The latter enzymes also contribute to the formation of triacylglycerols, although to a minor amount. Once steryl esters and triacylglycerols are built up, they can be stored in an organelle-like structure termed lipid droplet. In times of starvation or growth, these nonpolar lipids serve as important building blocks for membrane lipid formation and as energy pool. Therefore steryl esters and triacylglycerols need to be mobilized by hydrolytic enzymes. Whereas triacylglycerols are hydrolyzed by triacylglycerol lipases, our focus was set on the three steryl ester hydrolases Tgl1p, Yeh1p and Yeh2p. Tgl1p and Yeh1p are known to be lipid droplet resident enzymes, in contrast of Yeh2p which is located at the cell periphery. The overall aim of this work was to shed light on regulatory aspects of steryl ester metabolism. On the one hand, we investigated the steryl ester synthases Are1p and Are2p under conditions of deprived steryl ester mobilization. We clearly demonstrated a feedback regulation on the two steryl ester forming enzymes. Although gene expression and protein levels of the two acyltransferases were not affected in a strain lacking all three steryl ester hydrolases, the *in vitro* activity of Are1p and Are2p and the *in vivo* incorporation of radio-labelled fatty acids into steryl esters was significantly reduced under the tested conditions. On the other hand, we were curious about the fate of the three steryl ester hydrolases in the absence or presence of nonpolar lipids. For these studies we used strains either lacking steryl esters, triacylglycerols or lipid droplets at all. Based on the performed experiments we showed that Tgl1p and Yeh1p are retained to the endoplasmic reticulum solely in the strain lacking lipid droplets. As a consequence of this relocalization, the enzymes lost their hydrolytic activity and were highly unstable. Yeh2p was confirmed to be a protein localized at the plasma membrane, even in a strain lacking lipid droplets. Furthermore, Yeh2p is subject to a posttranslational modification, namely phosphorylation. In summary, results shown in this Thesis are one step forward to understand how steryl ester metabolism is regulated in the yeast *S. cerevisiae*.

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Generell Introduction

The fact that nonpolar lipids like triacylglycerols and steryl esters are crucial components serving as depot for membrane building blocks and energy storage is far beyond dispute. Additionally, sterol lipids are known to be indispensable to maintain membrane structural integrity and fluidity. Cholesterol, the main sterol in mammals, is on the one hand an essential precursor for the synthesis of vitamin D, bile acids and the biosynthesis of steroid hormones [1,2], on the other hand cholesterol functions in cell signalling and is involved in transport processes and nerve conduction [3]. Furthermore, disturbed homeostasis of cholesterol and cholesteryl esters can lead to human diseases such as Wolman disease, cholesteryl ester storage disease, the neurological diseases Alzheimer`s disease, Niemann-Pick type C and Tangier disease and to atherosclerosis [4–9], with its complications still presenting the leading death cause worldwide [10].

The yeast *Saccharomyces cerevisiae* is a well-established, highly appreciated and powerful eukaryotic model system to study lipid metabolism. Beside many other benefits, one considerable advantage of yeast includes the availability of gene-enzyme relationships in the pathways for lipid synthesis and turnover. However, there is still a lack of evidence concerning regulatory aspects in the network of lipid metabolism.

The present study aims to unravel regulatory principles of the steryl ester metabolism in the yeast *S. cerevisiae*. The review article in Chapter I ‘Steryl ester synthesis, storage and hydrolysis: A contribution to sterol homeostasis’ summarizes the process of synthesis and hydrolysis of steryl esters. Furthermore, the article pays particular attention to human diseases caused by a dysbalance in sterol and steryl ester homeostasis. Chapter II is entitled ‘Regulatory link between steryl ester formation and hydrolysis in the yeast *Saccharomyces cerevisiae*’. This part deals with the influence of the absence of all three steryl ester hydrolases of the yeast on the enzymes responsible for the formation of steryl esters. Results obtained throughout these investigations pointed towards a feedback regulation of steryl ester formation by steryl ester hydrolysis at the level of enzymatic activity. Chapter III ‘The impact of nonpolar lipids on the regulation of the steryl ester hydrolases Tgl1p and Yeh1p in the

yeast *Saccharomyces cerevisiae*' is dedicated to the fate of the two lipid droplet resident steryl ester hydrolases in the presence or absence of nonpolar lipids. Gene expression, protein level and stability as well as localization studies in combination with analyses of enzymatic activities indicated a strong crosstalk between the lack of nonpolar lipids and the lipid droplet localized steryl ester hydrolases. In Chapter IV entitled 'Yeh2p – the cell periphery localized steryl ester hydrolase in the yeast *Saccharomyces cerevisiae*' the effects of the lack of nonpolar lipids on the third steryl ester hydrolase are presented. We also demonstrate that the protein undergoes a posttranslational modification.

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CHAPTER I

Steryl ester synthesis, storage and hydrolysis: A contribution to sterol homeostasis

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Published in Biochimica et Biophysica Acta (BBA) – Molecular and Cell Biology of Lipids

Volume 1862, Issue 12, Pages 1534–1545, Epub 2017 Sep 6, Print 2017 Dec

doi: 10.1016/j.bbalip.2017.09.002

Abstract

Sterols are essential lipids of all eukaryotic cells, appearing either as free sterols or steryl esters. Besides other regulatory mechanisms, esterification of sterols and hydrolysis of steryl esters serve to buffer both an excess and a lack of free sterols. In this review, the esterification process, the storage of steryl esters and their mobilization will be described. Several experimental sytems are discussed but the focus was set on mammals and the yeast *Saccharomyces cerevisiae*. The contribution of imbalanced cholesterol homeostasis to several diseases, namely Wolman disease, cholesteryl ester storage disease, atherosclerosis and Alzheimer`s disease, Niemann-Pick type C and Tangier disease is described.

Highlights

- Sterols appear as either free sterols or steryl esters.
- Here we describe sterol esterification, storage and mobilization of steryl esters.
- Steryl ester hydrolases belong to the α/β-hydrolase superfamily.
- Steryl ester hydrolases occur in bacteria, fungi and mammalian cells.

Keywords: steryl ester synthase, steryl ester hydrolase, lipid droplet, sterol homeostasis

1. Introduction

Sterols are important organic compounds occuring in mammals, plants and fungi. They are absent from prokaryotes (bacteria and archaea) with *Mycoplasma* being one of only few exceptions [1]. The main sterol in mammals is cholesterol, whereas in the yeast *Saccharomyces cervisiae* ergosterol is the major sterol. In plants stigmasterol, sitosterol and campesterol are the most prominent components of this substance class. In vertebrates, hepatic cells typically produce the largest amounts of cholesterol. Cholesterol is a crucial structural component for membranes and essential to maintain both membrane structural integrity and fluidity. It has further functions in cell signalling, transport processes and nerve conduction [2]. Cholesterol is additionally essential as a precursor for the synthesis of vitamin D, bile acids and the biosynthesis of steroid hormones including the adrenal gland hormones cortisol and aldosterone, as well as the sex hormones progesterone, estrogens, and testosterone [3,4]. An unbalanced level of cholesterol and non-polar lipids including the esterified form of cholesterol, cholesteryl esters, is tightly linked to some human diseases. Disturbed homeostasis of cholesterol and cholesteryl esters can lead to obesity, diabetes, atherosclerosis, Wolman disease (WD), cholesteryl ester storage disease (CESD) and even neurodegeneration [5–10].

Sterols are synthesized in a multiple step process involving approximately thirty biochemical reactions. The basic structure of steroids is the gonane with its characteristic three fused cyclohexane rings and one cyclopentane ring. Common to all sterols is a hydroxyl group at the C atom in position 3 and the double bond at C-5,6. However, sterols from different kingdoms bear some minor modifications as shown in Figure 1 (for details see [11]).

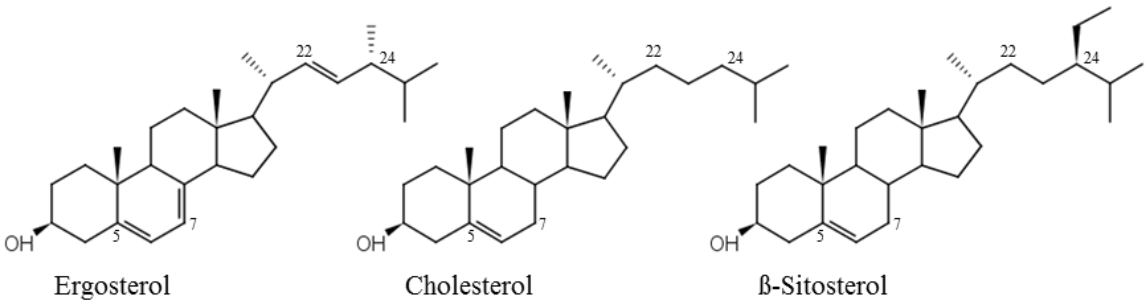

Figure 1 Structures of ergosterol, cholesterol and ß-sitosterol.

The major yeast sterol, ergosterol, has an additional double bond at position C-7 and C-22 and one methyl group at position C-24 compared to cholesterol, the predominant mammalian

sterol. The plant sterol ß-sitosterol resembles cholesterol, but shows a modified side chain at position C-24.

The entire sterol biosynthetic pathway can be devided into two sections. First, the pre-squalene pathway containing the mevalonate or isoprenoid pathway converts acetate to farnesyl pyrophosphate. Second, the oxygen dependent sterol biosynthesis pathway, commonly termed as post-squalene pathway, further converts farnesyl pyrophosphate through several reactions to, e.g. ergosterol in the yeast [11,12]. A common, although rather theoretical biosynthetic intermediate of ergosterol and cholesterol (since the downstream enzymes are working very efficiently) is cholesta-5,7,24(25)-trienol. In mammals, sterols are saturated at position C-7 and C-24. Therefore, the action of the dehydrocholesterol reducase 7 (DHCR7) and 24 (DHCR24) is essential. The endproduct of the yeast sterol biosynthetic pathway is ergosterol bearing a methyl group at position C-24, which is added by sterol C-24 methyl transferase (Erg6p). Furthermore, Erg5p, a sterol C-22 desaturase, introduces the double bond at position C-22 [11]. In contrast to mammals and fungi, cycloartenol is the starting point of almost all phytosteroids, but the triterpenoid squalene is a precursor of cycloartenol as well [13].

Besides other regulatory aspects, esterification of sterols and hydrolysis of steryl esters is a way to keep levels of both lipids constant and to fulfill cellular processes mentioned above. While steryl ester synthases are responsible for the formation of steryl esters, the hydrolases carry out the opposite reaction, namely the hydrolysis of steryl esters. Most of these hydrolyzing enzymes belong to the α/β-hydrolase superfamily. They have a conserved "catalytic triad" formed by histidine, the acidic amino acids aspartate or glutamate and a serine residue which is located in a highly conserved GXSXG sequence. Here, we will describe steryl ester synthases and steryl ester hydrolases from mammals, steryl ester forming enzymes from the plant *Arabidopsis thaliana* and both types of enzymes from the budding yeast *Saccharomyces cerevisiae*. Furthermore, we discuss six diseases tightly linked to imbalanced sterol homeostasis, namely Wolman disease, cholesteryl ester storage disease, atherosclerosis and Alzheimer`s disease, Niemann-Pick type C and Tangier disease.

2. Steryl ester synthases

Sterol ester (SE) formation is a normal homeostatic process, but it can also function as a buffering system for an excess of sterols. Moreover, steryl esters can also be regarded as storage form of fatty acids. Two types of enzymes catalyze the synthesis of SE, namely acyl-CoA cholesterol acyl transferase (ACAT; EC 2.3.1.26) also known as sterol O-acyltransferase (SOAT) or acyl-CoA:sterol acyltransferase (ASAT), and lecithin-cholesterol acyltransferase (LCAT, EC 2.3.1.43), also called phosphatidylcholine-sterol O-acyltransferase. Enzymes of the ACAT type using acyl-CoAs as co-substrates are intracellular proteins located in the endoplasmic reticulum. LCAT bound to high-density lipoproteins (HDL) and low-density lipoproteins (LDL) in the blood plasma uses a fatty acid from a phospholipid, preferentially from phosphatidylcholine (PC), to esterify a sterol [14,15].

In mammals, two ACAT isoenzymes, ACAT1 and ACAT2, encoded by two different genes were identified (for a recent review see [16]). Both ACATs play important roles in cellular cholesterol homeostasis in various tissues. Human ACAT1 (hAcat1) is located to chromosomes 1 and 7 [17]. In human tissues, four ACAT1 mRNAs with sizes estimated at 7.0, 4.3, 3.6 and 2.8 kb are present. Due to alternative splicing, three hAcat1 mRNAs are translated to a single 50 kDa protein representing the major ACAT1 in various human cells and tissues [18]. A minor ACAT protein in human macrophages of 56 kDa whose function is currently unknown is derived from the 4.3 kb Acat1 mRNA [19]. The 50 kDa protein of ACAT1 occurs as a homotetramer in intact cells and *in vitro* [20]. Its membrane topology in living cells is controversial as five to nine transmembrane domains were suggested [21–23]. The discrepancy may be caused by the different methods of analysis employed. ACAT1 appears to have two functional sides, one being responsible for subunit interaction to form an ACAT1 dimer, and the other representing the substrate binding and/or catalysis site [24]. The conserved domain FYXDWWN (aa 403-409) appears to be involved in fatty acyl-CoA binding [25]. Furthermore, H460, S456 and D400 also seem to be essential for enzyme activity [26]. ACAT1 may also contain thioesterase activity through the classical Ser/Asp/His catalytic triad.

ACAT2 identified through homology to ACAT1 [27–29] is a 46 kDa protein and enzymatically related although not identical to ACAT1 [18,30]. ACAT2 is also an integral

membrane protein. Two to five transmembrane domains and several additional stretches embedded in the membrane were suggested [23,31].

In humans, ACAT1 expressed in many different tissues and cell types, accounts for the major ACAT activity *in vitro* [18,32–34]. By contrast, ACAT2 is predominantly expressed in liver and intestine [27–29]. Parini *et al.* (2004) demonstrated the presence of both ACAT1 and ACAT2 in adult human hepatocytes. ACAT1 activities were relatively constant whereas ACAT2 enzymatic activities varied among individual human liver samples and between species (human and monkey liver microsomes). mRNA levels of ACAT1 are considerable higher than those of ACAT2 [35]. Thus, the expression of ACAT1 appears to be constitutive whereas that of ACAT2 might be inducible. Similar observations were made with various animal models [18,34,36,37]. Both ACAT1 and ACAT2 contribute to the synthesis of cholesteryl esters stored in the core of VLDL (very low-density lipoproteins) [38–44].

DGAT1 is an enzyme that belongs besides ACAT1 and ACAT2 to the DGAT1 gene familiy; all proteins are members of the membrane-bound O-acyltransferase (MBOAT) superfamily [45]. DGAT1 was identified by sequence similarity to ACAT1 and ACAT2. DGAT1 shows diacylglycerolacyltransferase activity, the enzyme activity for the ultimate step in triacylglycerol formation [27–29,46–48]. DGAT2 was identified to posses diacylglycerolacyltransferase activity, too [49]. However, this enzyme belongs to a different gene family, namely the DGAT2 familiy which includes acyl-CoA:monoacylglycerol acyltransferase-1 (MGAT1) [50], MGAT2 [51,52], MGAT3 [53] and wax monoester synthases [54–56].

Lecithin-cholesterol acyltransferase (LCAT) synthesizes cholesteryl esters by esterification of free cholesterol with a fatty acid set free from the *sn*-2 position of phosphatidylcholine (PC) [57,58]. Cholesteryl esters are then sequestered into the core of a lipoprotein particle. The primary site of LCAT reaction is on circulating high-density lipoproteins (α-LCAT activity), but activity was also detected in apolipoprotein (apo) B100-containing particles (ß-LCAT activity) [59]. LCAT is synthesized on ER bound ribosomes, N- and O-linked glycosylated to become enzymatically active and secreted. On the surface of high-density lipoproteins (HDL) LCAT mediates esterification of cholesterol taken up from peripheral tissue cells [60]. LCAT is a key player in HDL biogenesis and mutations result in profound HDL deficiency. Complete LCAT deficiency results in loss of mature HDL and

accumulation of pre-β-HDL [61]. β-LCAT activity located on apoB-containing lipoproteins affects the cholesterol to cholesteryl ester ratio and the phospholipid level in total plasma [62].

Studies addressing the role of LCAT in atherosclerosis performed with loss-of-function mutations and with large cohorts in population led to controversial results [63–66]. In a mix-gender analysis, there was no correlation of plasma LCAT concentration and the risk of coronary artery disease [67]. For men, however, the risk seemed to be negatively associated with the LCAT level, whereas the opposite effect was observed with women. In the coronary cases LCAT activity was increased compared to controls probably as a protective effect of HDL cholesterol [68]. In a more recent study of men and women at high risk of atherosclerotic heart disease there was no obvious correlation of plasma LCAT and atherosclerosis [69]. Also in animal models especially with transgenic mice the role of LCAT in atherosclerosis remained contradictory [70–77].

In plants, biochemical studies suggested that phospholipids and/or neutral lipids could serve as acyl donors for SE synthesis [78,79]. In *Arabidopsis thaliana*, the phospholipid:sterol acyltransferase gene AtPSAT1 (At1g04010) was identified by homology to the mammalian LCAT and biochemically characterized [80,81]. The ER located enzyme transfers unsaturated fatty acids from the *sn*-2 position of phosphatidylethanolamine and phosphatidylcholine to sterols. Preferred acceptor molecules of AtPSAT1 are campesterol and sitosterol as well as cholesterol, a minor biosynthetic end product in *Arabidopsis*. In coincubation experiments the sterol precursors cycloartenol or obtusifoliol were efficiently acylated when sitosterol was present [80]. Bouvier-Navé (2010) reported on the involvement of AtPSAT1 in leaf senescence, its essential role in free sterol homeostasis and its contribution to SE formation in leaves and seeds. As another *Arabidopsis* sterol acyltransferase, AtASAT1 (At3g51970) was identified which is structurally related to the animal and yeast ACATs. This enzyme prefers saturated fatty acyl-CoAs as acyl donors and cycloartenol as acceptor molecule [82].

In the yeast, *Saccharomyces cerevisiae*, two acyl-CoA:sterol acyltransferases named Are1p and Are2p (ACAT related enzymes) [83,84] were identified. In an *are1*Δ*are2*Δ double mutant sterol esterification is completely abolished indicating that the respective gene products are the only SE synthesizing enzymes in the yeast. Interestingly, growth of the *are1*Δ*are2*Δ double mutant was not affected under the tested conditions [83,84]. Are1p and

Are2p share sequence identity of 49%, and approximately 24% to human ACAT. Are2p catalyzes the major yeast ACAT enzyme activity as estimated by *in vitro* assays. Both Are-enzymes are localized to the endoplasmic reticulum. Regarding substrate specificity, Are1p esterifies ergosterol as well as precursor sterols, particularly lanosterol [85]. It was assumed that the reason for the esterification of ergosterol precursors is that sterol intermediates from SE can be rapidly converted to ergosterol upon requirement [86]. Are2p has a preference for ergosterol as a substrate thus forming an important sterol pool for membrane formation [85]. Polakowski *et al.* (1999) did not observe a noticeable increase in esterified sterols when *ARE1* was overexpressed probably due to substrate limitation [87]. Jensen-Pergakes (2001) observed an accumulation of sterol intermediates, specifically lanosterol and zymosterol, upon *ARE1* overexpression [88].

Studies of regulatory aspects addressed the influence of oxygen on the expression of the two yeast Are enzymes. Are1p is the hypoxic, oxygen deprived form, whereas Are2p represents the major aerobic activity [89]. Transcriptional regulation of *ARE* genes combined with the substrate specificities of Are1p and Are2p may contribute to the adaptation of yeast sterol metabolism in hypoxia. Recently, it was shown that yeast Are2p is also subject to regulation by oleate present in the medium [90]. This effect was not due to decreased expression of *ARE2* but due to competitive enzymatic inhibition of Are2p by free oleate. Deletion of *ARE1* and *ARE2* also influences total sterol synthesis in yeast [91]. In an *are1*Δ*are2*Δ double mutant expression of *ERG3* encoding a C-5 sterol desaturase [92] is decreased. In such strains, the amount of the squalene epoxidase Erg1p is also lowered especially in the lipid droplet fraction [93]. It appears that Erg1p becomes instable under these conditions. Moreover, incorporation of ergosterol into the plasma membrane was reduced in an *are1*Δ*are2*Δ mutant [93]. Deletion of *ARE1* and *ARE2* leads to synthetic lethality by an additional mutation in the *ARV1* (*ARE2* required for viability) gene [94]. Involvement of Arv1p in sterol trafficking was suggested, especially in sterol exit from the endoplasmic reticulum which becomes important in the absence of the neutralizing effect of sterol esterification [88]. Consistent with a role of Arv1p in sterol translocation, *arv1*Δ mutants displayed changes in the intracellular sterol distribution and were defective in sterol uptake [94].

Table 1 summarizes the above mentioned enzymes responsible for steryl ester formation, thzeir occurrence and their most important biochemical features.

Table 1 Most prominent steryl ester synthases in mammals, plants and the yeast *Saccharomyces cerevisiae*, as a representative of the kingdom of fungi.

ACAT = acyl-CoA:cholesterol acyltransferase, LCAT = lecithin:cholesterol acyltransferase, VLDL = very low-density lipoproteins, IDL = intermediate density lipoproteins, LDL = low-density lipoproteins, HDL = high-density lipoproteins, PC = phosphatidylcholine, PE = phosphatidylethanolamine

Organism		*Enzyme*	*Type of Enzyme*	*Special features*	*Co-Substrate*
Mammals	Humans	ACAT1	ACAT	Major ACAT enzyme in humans; constitutively expressed in many different tissues; synthesis of cholesteryl esters stored in the core of VLDL	Acyl-CoAs
		ACAT2	ACAT	Inducible; expressed in liver and intestine; synthesis of cholesteryl esters stored in the core of VLDL	Acyl-CoAs
		LCAT	LCAT	α-LCAT activity: primary site of reaction is on circulating HDL -> key player in HDL biogenesis. β-LCAT activity: the enzyme acts on apoB 100 containing lipoprotein particles (VLDL,IDL, LDL)	Fatty acids from the *sn*-2 position of PC
Plants	*Arabidopsis thaliana*	AtASAT1	ACAT	Acylates cycloartenol	Saturated fatty acyl-CoAs
		AtPSAT1	LCAT	Main substrates are campesterol and sitosterol; cholesterol, cycloartenol, obtusifoliol	Unsaturated fatty acids from the *sn*-2 poition of PE and PC

Fungi	*Saccharomyces cerevisiae*	Are1p	ACAT	Hypoxic form of ACAT; uses ergosterol and precursors of ergosterol, particularly lanosterol	Acyl-CoAs
		Are2p	ACAT	Major enzyme under aerobic conditions; main substrate is ergosterol	Acyl-CoAs

After synthesis, the SEs together with triacylglycerols (TGs) are stored in specialized compartments. The lipid storage compartment within the cell are the so-called lipid droplets, also known as lipid particles, oil droplets, lipid bodies, oil bodies or oleosomes in mammals and plants, respectively. In this review article, the biochemistry, molecular and cell biology of lipid droplets will not be discussed in detail, but the reader is referred to recent review articles describing the nature of lipid droplets in various cell types [95–99]. As a common structural feature, the hydrophobic core of lipid droplets is surrounded by a phospholipid monolayer membrane which is equipped with distinct proteins. In most mammals, lipid droplets are rich in TGs. In the yeast *S. cerevisiae*, lipid droplets contain approximately equal amounts of TGs and SEs [100]. Lipid droplets are believed to originate from ER membranes (for recent reviews see [97,99]).

Within mammalian organisms, sterols can be transported in the form of SE packed in lipoproteins. Lipoproteins are classified by their density into chylomicrons, very low-density lipoproteins (VLDL), intermediate density lipoproteins (IDL), low-density lipoproteins (LDL) and high-density lipoproteins (HDL). Here, we will only briefly mention the contribution of lipoprotein particles to sterol/SE storage. Chylomicrons, which are the largest and least dense of the lipoproteins, contain ~3% cholesteryl esters and ~1% free cholesterol. In VLDL, 12-15% cholesteryl esters and 8-10% cholesterol accumulate. IDL which is smaller than VLDL contains 32-35% cholesteryl esters and 8-10% cholesterol. LDL (“bad cholesterol”) is smaller than IDL and harbors 37-48% cholesteryl esters and 8-10% cholesterol. Finally, HDL (“good cholesterol”) contains 15-30% cholesteryl esters and 2-10% cholesterol. As a general structural feature, lipoproteins similar to cytoplasmic lipid droplets contain a core of non-polar lipids surrounded by a phospholipid surface layer with proteins embedded. Structural and biological features of lipoproteins have been summarized in a number of reviews [101–108].

3. Mobilization of sterols from nonpolar lipid pools

Steryl ester hydrolases cleave steryl esters yielding sterols and free fatty acids. Structurally most of these enzymes belong to the α/β-hydrolase superfamily. They have a conserved "catalytic triad" formed by the amino acids histidine, an aspartate or glutamate and a serine residue which is located in a highly conserved GXSXG sequence.

In mammals, some steryl ester hydrolytic enzymes have been identified (for recent reviews see [109,110]). Ghosh *et al.* [111] described a neutral cholesteryl ester hydrolase (CEH) in human macrophages named Ces1. Transfection of this cDNA in COS-1 and COS-7 cells resulted in increased cholesteryl ester hydrolytic activity. The enzyme is localized to the surface of lipid droplets in lipid-laden cells and mobilizes cholesteryl esters from the droplets [112,113]. Overexpression of Ces1 in macrophages resulted in an increased rate of cholesterol efflux to ApoA1, HDL and serum [114]. Crow *et al.* [115] demonstrated accumulation of cholesteryl esters in macrophages by inhibition of CEH. Taken together, these data support the role of this enzyme in regulating the content of cholesteryl esters and the cholesterol efflux. The mouse ortholog of Ces1 has been named Ces3/TGH, but cholesteryl ester hydrolase activity was not shown for this protein. Two other enzymes, Es22/Egasyn (mouse) [116] and AADA (mouse/human) [117–119] showed low steryl ester hydrolase activity.

Another mammalian steryl ester hydrolase is KIAA1363/NCEH1/AADACL1 which was initially identified as enzyme catalyzing the hydrolysis of 2-acetyl-1-alkylglycerol [120]. Mice lacking KIAA1363/NCEH1/AADACL1 accumulated cholesteryl esters in macrophages and exhibited a decrease in cholesteryl esterase activity [121]. Overexpression of KIAA1363/NCEH1/AADACL1 in macrophages markedly decreased the cholesteryl ester content [122]. Involvement of KIAA1363/NCEH1/AADACL1 in macrophage cholesteryl ester metabolism has recently been challenged by a study which did not confirm the above mentioned findings suggesting the existence of (an)other, as yet unidentified cholesteryl ester hydrolase(s) in macrophages [123].

Lysosomal acid lipase (LAL), a 46 kDa glycoprotein encoded by the *LIPA* gene on chromosome 10q23.2-23.3 [124], is the primary enzyme responsible for the hydrolysis of cholesteryl esters from LDL and modified LDL. The home organelle of LAL is the lysosomal compartment [125,126]. More than 40 mutations in the *LIPA* gene are known to contribute to

severe disease manifestation [127]. The most common mutation in patients with cholesteryl ester storage disease (CESD) is a splice junction mutation at exon 8 of *LIPA* (E8SJM). As a result, correctly spliced LAL protein and activity is reduce to about 3 – 5% [128–130]. A recent study by Grumet *et al.* [131] revealed the ability of murine LAL to hydrolyze retinyl esters, whose activity could be even stimulated in the presence of the anionic phospholipid *sn*-1;*sn*-1' bis(monoacylglycero)phosphate (BMP).

Currently, enzyme/s responsible for the hydrolysis of steryl esters in the plant *Arabidopsis thaliana* is/are not known.

In the yeast *Saccharomyces cerevisiae,* the three steryl ester hydrolases Yeh1p, Yeh2p and Tgl1p were identified [132,133]. Tgl1p and Yeh1p are lipid droplet resident proteins, whereas Yeh2p localizes to the plasma membrane. Surprisingly, the highest activity was found to be associated with the plasma membrane. In a *yeh2*Δ deletion strain the amount of ergosteryl esters was reduced. The two other yeast SE hydrolases Tgl1p and Yeh1p were identified as paralogues of mammalian acid lipases [133]. Both enzymes localize to lipid droplets, but their steryl ester hydrolase activity is low [133,134]. Köffel *et al.* [133] showed that Yeh1p is more active under anaerobiosis which is mimicked by heme deficiency in aerobically cultivated cells. Ploier *et al.* [135] reported on a regulatory link between SE formation and hydrolysis. A strain deficient in all three steryl ester hydrolases showed a reduced *in vitro* activity of the enzymes forming SE. The *in vivo* incorporation of radiolabelled activated fatty acids into steryl esters was also less efficient in the triple mutant compared to wild-type cells. In the yeast *S. cerevisiae* the maintenance of sterol homeostasis can additionally be achieved via an acetylation/deacetylation cycle. Sterol acetylation is mediated by the acetyltransferase Atf2 [136]. Acetylated sterols further are subject for secretion. Thus, sterol acetylation can be considered as a detoxification mechanism in the yeast to circumvent a sterol overload. The gene product of *SAY1* is a membrane-anchored deacetylase in the ER and required for sterol deacetylation [137]. Lack of *SAY1* results in vesicle mediated export of acetylated sterols to the culture medium.

Table 2 summarizes the properties of the steryl ester hydrolases desribed in this section. Regarding steryl ester hydrolases from other fungi like *Fusarium oxysporum, Candida rugosa, Ophiostoma piceae, Melanocarpus albomyces* and *Trichoderma* sp, we refer to a current review by Vaquero *et al.* [138]. This article also contains a section about steryl

ester hydrolases in bacteria ranging from *Pseudomonas, Burkholderia, Streptomyces* and *Acinetobacter* to *Chlamydia.*

Table 2 Prominent steryl ester hyrolases in mammals and the yeast *Saccharomyces cerevisiae*, as a representative of the kingdom of fungi.

HDL = high desity lipoprotein, LAL = lysosomal acid lipase, WD = Wolman disease, CESD = cholesteryl ester storage disease, LDL = low-density lipoprotein

Organism		*Enzyme*	*Type of Enzyme*	*Special features*	*Substrate*
Mammals	Humans	Ces1	α/β-hydrolase superfamily	The cholesteryl ester hydrolase (CEH) has an important role in regulating the content of cholesteryl esters and cholesterol efflux to ApoA1 and HDL.	Cholesteryl esters
		LAL		Lysosomal acid lipase is active in lysosomes; associated diseases: WD, CESD	Cholesteryl esters from LDL and modified LDL
	Humans/Mouse	KIAA1363/ NCEH1/ AADACL1		Controversioal results regarding cholesteryl ester hydrolysis in macrophages	2-acetyl-1-alkylglycerol
		AADA		Low steryl ester hydrolase activity	Steryl esters
	Mouse	Es22/Egasyn		Low steryl ester hydrolase activity	Steryl esters

Fungi	*Saccharomyces cerevisiae*	Yeh1p	α/β-hydrolase superfamily	Lipid droplet resident protein; upregulated under anaerobic conditions	Ergosteryl esters; fecosteryl esters
		Yeh2p		Plasma membrane resident protein; highest activity under aerobic conditions	Mainly ergosteryl esters
		Tgl1p		Lipid droplet resident protein	Ergosteryl esters; zymosteryl esters

4. Diseases related to steryl ester metabolism and sterol homeostasis

A disbalance in sterol homeostasis can lead to several severe diseases in humans. In the following, the contribution of sterol homeostasis to six diseases will be described, namely Wolman disease, cholesteryl ester storage disease, atherosclerosis and Alzheimer`s disease, Niemann-Pick type C and Tangier disease.

4.1. Lysosomal acid lipase deficiency (LAL-D): Wolman disease (WD) and cholesteryl ester storage disease (CESD)

Lysosomal acid lipase deficiency (LAL-D) is a rare, autosomal recessive, lysosomal storage disorder. The characteristic of this disease is the absence or deficiency of the lysosomal acid lipase gene *(LIPA)*, whose gene product is responsible for the catabolism of cholesteryl esters and triglycerides derived from LDLs. The pathological manifestations of this disorder are lysosomal accumulation of cholesteryl esters and to a lesser extent also triglycerides mainly in hepatocytes, adrenal glands, intestine and cells of the macrophage-monocyte system throughout the body. Hallmarks of LAL-D are hepatomegaly, splenomegaly, elevated serum total cholesterol, LDL-cholesterol, triglycerides and transaminases (aspartate aminotransferase AST, alanine aminotransferase ALT), microvesicular steatosis, which leads to fibrosis, micronodular cirrhosis and ultimately to liver failure [139–148]. LAL-D can be divided into two major phenotypes: LAL-D present within the first year of life is termed Wolman disease (WD) [139,149], whereas the disease with late-

onset present in children and adults is known as cholesteryl ester storage disease (CESD) [150–153].

As lysosomal acid lipase (LAL) is the only lipase within lysosomes responsible for hydrolysis of cholesteryl esters and triglycerides, this enzyme plays a crucial role in lipid homeostasis [154,155]. Comparing the cellular processes shown in Figure 2, the dramatic accumulation of cholesteryl esters in lysosomes in LAL-D patients becomes clearly evident due to either diminished or absence of cholesteryl ester hydrolysis. In healthy individuals, free cholesterol is transported after cholesteryl ester (CE) cleavage to the cytosol. However, in LAL-D patients, the decreased amount of cytosolic cholesterol results in a compensatory upregulation of cholesterol synthesis and endocytosis via an increased number of LDL receptors [156,157]. In disease patients, the production of VLDL-C (very low-density lipoprotein cholesterol) is increased [158], and due to a dysregulation of the ATP binding cassette transporter 1 (ABCA1), less cholesterol can be passed on to HDL particles [159].

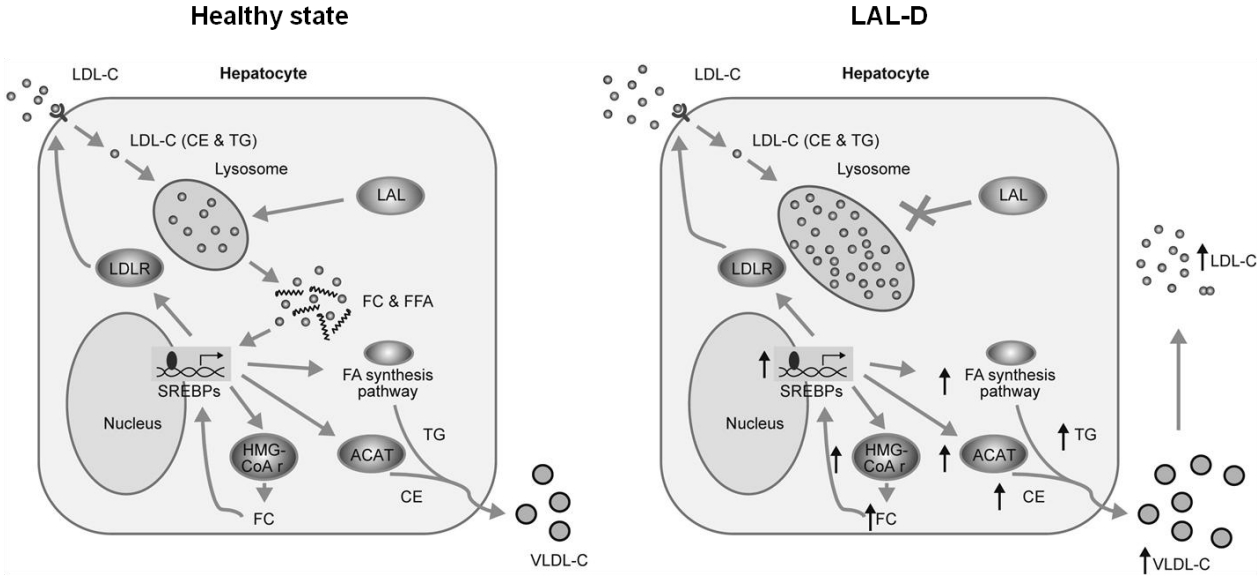

Figure 2 modified from Reiner *et al.* [160]. Cellular cholesterol homeostasis in healthy individuals and patients with LAL-D.

In healthy individuals, LAL is responsible for the cleavage of CE and TG in the lysosome. The resulting products are transported to the cytoplasm. Free cholesterol (FC) can interact with the transcription factor SREBP to modulate intracellular cholesterol production. High free intracellular amounts of cholesterol lead to a downregulation of LDL receptors, inhibition of HMG-CoA r and stimulation of ACAT. In LAL-D patients, CE and TG accumulate in

lysosomes causing a compensatory upregulation of HMG-CoA r activity, enhanced endocytosis of LDL-C via LDL receptors and increased production of VLDL-C. ACAT = acyl-CoA:cholesterol acyltransferase; CE = cholesteryl ester; FA = fatty acid; FC = free cholesterol; FFA = free fatty acid; HMG-CoA r = 3-hydroxy-3-methyl-glutaryl-CoA reductase; LAL = lysosomal acid lipase; LAL-D = lysosomal acid lipase deficiency; LDL-C = low-density lipoprotein cholesterol; LDLR = low-density lipoprotein remnant; SREBPs = sterol regulatory element binding proteins; TG = triglycerides; VLDL-C = very low-density lipoprotein cholesterol

In respect to cholesterol homeostasis, characteristics of LAL-D are elevated total serum cholesterol, elevated LDL-C (low-density lipoprotein cholesterol) and low HDL-C (high-density lipoprotein cholesterol). Just recently, sebelipase alfa was approved for the treatment of LAL-D [161–164]. Sebelipase alfa is undoubtedly a very potent and promising recombinant human enzyme replacement, showing significant improvement in biochemical parameters for late-onset disease. Yet, it is mandatory to monitor the long-term outcome of this enzyme replacement therapy.

4.2. Atherosclerosis

Atherosclerosis is a multifactoral, chronic, immune-inflammatory, complex disease with its complications still presenting the leading death cause worldwide [165]. Generally spoken, in atherosclerosis lipid metabolism is disturbed which goes along with cholesterol accumulation in the vascular walls and plaque formation [166]. Key players in atherosclerosis are macrophages [167–171]. Pro inflammatory stimuli in the vascular endothelium lead to attached monocytes. Then, these monocytes penetrate to the arterial intima, the destination of differentiation to macrophages, which are able to take up oxidized LDL (ox-LDL) by so-called scavenger receptors [172–174]. Ox-LDL is delivered to late endosomes/lysosomes. In the latter organelle, cholesteryl esters derived from ox-LDL can be first hydrolyzed by LAL yielding free cholesterol and free fatty acids [154,155]. After the transport to the cytosol, free cholesterol can be esterified by ACAT1 in the endoplasmic reticulum [175]. CE are stored within lipid droplets and after a second hydrolysis mediated by nCEH and NCEH1, free cholesterol is ready to be transported out of macrophages by cholesterol ATP-binding cassette (ABC) transporters [176–178]. HDL or ApoA1 then capture the released cholesterol and

conduct the reverse cholesterol transport to the liver [179] (See Figure 3). Central to the development of atherosclerosis is an increased uptake of oxLDL, accumulation of esterified cholesterol in the cytoplasm of macrophages and ultimately the transformation of these macrophages into lipid-laden foam cells, typical cells in the atherosclerotic plaque [180]. In addition, macrophagial overload with free cholesterol could lead to the formation of highly toxic cholesterol crystals [181]. As a result, the intracellular cholesterol metabolism is influenced and inflammatory cytokines are released [182].

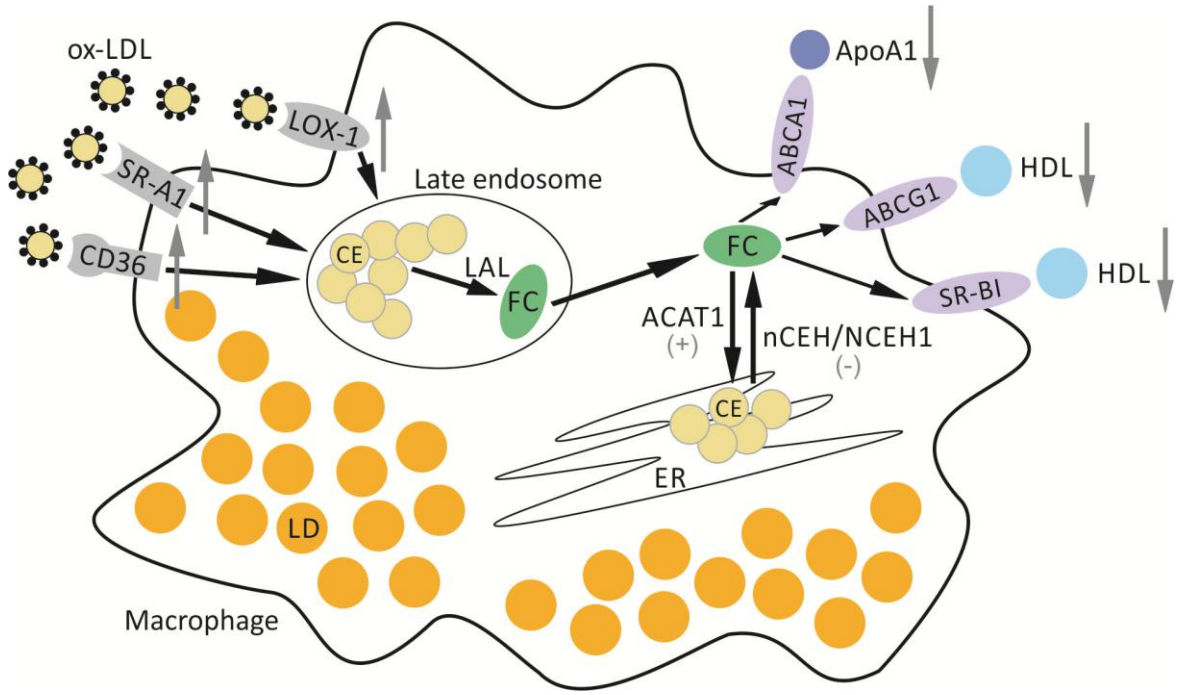

Figure 3 Foam cell in atherosclerosis.

Under atherogenic conditions, the uptake of oxidized low-density lipoprotein (ox-LDL) via the scavenger receptors (LOX-1, SR-A1 and CD36) is increased. In late endosomes, cholesteryl esters (CE) derived from ox-LDL are degraded by lysosomal acid lipase (LAL) to free cholesterol (FC) and fatty acids. FC gets transported to the cytosol and esterified in the endoplasmic reticulum (ER) via ACAT1 in order to yield CE which are stored within lipid droplets (LDs). Subsequent to a second hydrolysis mediated by nCEH/NCEH1, FC can be transported out of macrophages by cholesterol ATP-binding cassette (ABC) transporters (ABCA1, ABCG1 and SR-BI). High-density lipoprotein (HDL) and ApoA1 take up the released cholesterol and conduct the reverse cholesterol transport to the liver. Accumulation of CE laden LDs in macrophages prompt the formation of foam cells.

To study the pathogenesis of atherosclerosis and to find potential treatment strategies, several transgenic or knock out mouse models were established. The two most used mouse models in atherosclerosis research are ApoE-deficient mice ($ApoE^{-/-}$) and mice deficient in the receptor for LDL ($LDLR^{-/-}$). Zhang *et al.* [183] successfully deleted the mouse apolipoprotein E *(apoE)* gene. ApoE-deficient mice ($ApoE^{-/-}$) suffer from severe hypercholesterolemia and develope extensive spontaneous atherosclerotic lesions even on chow diet. Ishibashi *et al.* [184] generated a mouse deficient in the receptor for LDL ($LDLR^{-/-}$). These mice represent a more moderate model than the $ApoE^{-/-}$ mouse, mainly because of the milder degree of hyperlipidemia [185,186]. On chow diet, plasma cholesterol is only modestly elevated and rapid development of atherosclerosis can be achieved just by high fat cholesterol (HFC) diet in $LDLR^{-/-}$ mice [187–189]. Each single model bears its own characteristics and limitations in terms of learning how to treat atherosclerosis. For a detailed overview of mouse models and other animals used in atherosclerosis research, the reader is referred to the literature [183–185,187,190–199].

4.3. Neurological disorder: Alzheimer`s disease (AD)

Alzheimer´s disease (AD) is a multifactorial neurodegenerative disease, highly related to age, showing ß-amyloid (Aß) accumulation forming extracellular senile plaques, hyperphosphorylation of the tau protein and neuronal loss accompanied by proliferation of astrocytes. Due to the fact, that the production of Aß from amyloid precursor protein (APP) is enhanced when converting free cholesterol to CE, the importance of ACAT enzyme in the development of AD can be stressed [200]. In the literature there is clear evidence, that hypercholesterolemia is one of the risk factors of AD. In mammals, the brain harbors approx. 25% of total body cholesterol although the brain makes up less than 10% of total body mass [201]. All cholesterol is synthesized within the brain and is particularly abundant in myelin. The brain deals with cholesterol overload by conversion into 24-hydroxycholesterol. The latter compound can cross the blood-brain barrier and is transported to the liver for excretion into the bile [202,203]. The major lipoprotein in the central nervous system (CNS) is ApoE. ApoE is synthesized mainly by astrocytes and is responsible for the cholesterol transport in the CNS [204–206]. In humans, there are three common alleles of ApoE: ApoE2, ApoE3 and ApoE4. Carriers of the ApoE ε4 allele are more susceptible to AD mainly due to enhanced Aß aggregation [207–211].

Studies revealed that plasma cholesterol levels were elevated in AD patients [212]. Treatment by lipid-lowering therapy helped to reduce the risk of the neurodegenerative disease [213–216]. Furthermore, the blood-brain barrier (BBB) serves as a kind of protective barrier to avoid entry of neurotoxic plasma components to the CNS. Reduced production of tight junction proteins due to high-cholesterol diet in rabbits led to disrupted integrity of the BBB. Dysfunction of the BBB is associated with the development and progression of AD [217,218]. In case of increased intracellular cholesterol levels, the proportion of cholesterol found in so-called lipid rafts in the plasma membrane is enhanced, too. As a result, APP binding to the cholesterol in the membranes is triggered, hence also the production of Aß [219]. Grimm *et al.* [220] reported enhanced activities of the enzymes responsible for anabolism of Aß when intracellular cholesterol levels are increased. To list all causes that go along with AD would be far beyond the scope of this article; however, the above-mentioned findings highlight the influence of imbalanced cholesterol metabolism in AD.

4.4. Neurological disorder: Niemann-Pick type C (NPC) disease

Niemann-Pick type C (NPC) disease is an autosomal recessive lysosomal lipid storage disorder with its characteristic of accumulation of unesterified cholesterol and other lipids (sphingomyelin, sphingosine and gangliosides) within the late endosomes/lysosomes. Pathological manifestations in humans and mice are progressive neurodegeneration and hepatosplenomegaly leading to premature death. The reasons for the accumulation of cholesterol in NPC disease are mutations in either of two lysosomal proteins, Niemann-Pick C1 (NPC1) or Niemann-Pick C2 (NPC2). Carstea *et al.* [221] were the first to identify NPC1 by positional cloning. NPC1 is a lysosomal transmembrane glycoprotein with 13 putative transmembrane helices. More than 200 known mutations result in NPC disease [222–224]. A 95% majority of NPC disease results from mutations in the NPC1 gene. The small soluble glycoprotein NPC2, previously studied as HE1, was identified by Naureckiene *et al.* [225] from a proteomic screening of mannose-6-phosphorylated proteins. NPC2 accounts for roughly 5% of disease cases. The lysosomal protein has the ability to bind cholesterol in a 1:1 stoichiometry [226] and transfers the ligand to membranes very rapidly [227]. How NPC1 and NPC2 interact with each other is not yet completely understood. However, there is strong evidence for functional cooperativity, perhaps in the way of sequentially binding the same ligand [228]. Some studies propose a directly transfer of cholesterol from NPC2 within the

lysosome to the luminal N-terminal domain of NPC1 [229–231]. Nevertheless, direct protein-protein interaction of NPC1 and NPC2 has not been observed.

4.5. Neurological disorder: Tangier disease (TD)

In humans, 49 different genes are known to encode ABC (adenosine triphosphate (ATP)-binding cassette) transporters. These ABC transporters are organized in the seven subfamilies ABCA – ABCG [232,233]. All of the 12 human ABCA transporters are full-size transporters consisting of a single polypeptide containing at least two TMDs (transmembrane domains) with multiple membrane-spanning segments and two NBDs (nucleotide-binding domains) [232,234]. Several mutations causing the lack of functional ABCA1 transporter are associated with the very rare autosomal recessive disorder called Tangier's disease, also known as familial HDL deficiency [235,236]. Tangier disease was first described by Fredrickson *et al.* [237]. Patients suffering from TD are hindered in reverse cholesterol transport (RCT) and show very low to almost absent HDL, apolipoprotein A1 (ApoA1) – known to be the major HDL apolipoprotein – low total plasma cholesterol, moderately elevated triglyceride and higher incidence of premature cardiovascular disease. TD causes neuropathy in half of the affected individuals. Due to the defective cholesterol and lipid transport, accumulation of cholesteryl esters in different reticuloenothelial cells of various tissues like tonsils, lymph nodes, bone marrow, thymus, spleen, liver and intestinal mucosa as well as in fibroblasts, smooth muscle cells and Schwann cells could be observed [238]. ABCA1 plays a major role in the first step of RCT resulting in the transfer of free cholesterol from peripheral cells to lipid-poor apoA1 [239–241]. In order to maintain the release of nascent HDL via ApoA1 lipidation through ABCA1, the ABCA1 transporter has to be localized to the plasma membrane and fulfill three distinct activities: i) ABCA1 must be able to remodel the plasma membrane via its PS (phosphatidylserine) floppase activity, ii) ABCA1 must be able to bind to ApoA1 and iii) ABCA1 must mediate the unfolding of the N-terminal domain of ApoA1 [242–254].

5. Summary and Conclusion

Sterols are important components of biomembranes, but also precursors of hormones required for cell signaling. They occur in two forms, namely as free sterols and as esterified

sterols. Esterification of sterols helps to keep the level of free sterols balanced. Sterol homeostasis is a crucial process in all eukaryotic cells.

In this review article we described sterol esterification, storage and hydrolysis in different cellular systems. Most enzymes involved in this processes have been identified and characterized. As shown in Table 1 sterol acyltransferases can be devided in two groups according to their enzymatic properties, namely in the ACATs and the LCATs. Depending on the organism studied one or both groups have been detected. Steryl ester hydrolases belong to the α/β-hydrolase superfamily (see Table 2). They possess a conserved "catalytic triad" formed by the amino acids histidine, an aspartate or glutamate and a serine residue. Also the organelles involed, especially the storage compartment of steryl esters, the lipid droplets were recently studied intensively. In the core of lipid droplets steryl esters are stored together with the other storage lipid, the triacylglycerols.

While enyzmes of the sterol homestasis pathways have been characterized little is known about regulation of these processes. It is not exactly known how overall formation and degradation of steryl esters is regulated. Also the regulatory aspects of enzymes with overlapping function among each other need to be clarified. Another important step in storage lipid metabolism is their deposition in lipid droplet. It is not exactly known how steryl esters and also triaclyglcerols get to their site of storage and how they leave the lipid droplet compartment. Furthermore, the link of storage lipid metabolism to membrane formation and degradation is an aspect which is not well studied. This route is important for a balanced cell structure.

Another aspect which should be studied in more detail is the involvement of steryl ester formation and degradation in human diseases like the Wolman disease, cholesteryl ester storage disease, atherosclerosis and even Alzheimer`s disease. As an example, it is not clear whether the mammalian KIAA1363 is indeed a cholesteryl ester hydrolase in macrophages or not. More detailed studies of the metabolism and also of its regulation will be required to shed more light on the link between steryl ester metabolism and the diseases mentioned above.

Finally, the crystal structure of the steryl ester synthases and steryl ester hydrolases may be helpful to understand their function. Such studies may be important for a better knowledge of the enzymology and the regulation of steryl ester homeostasis. Moreover, the

exact structure of the enzymes, especially of those who are disease related, may be valuable for drug development. However, such studies are difficult as purification of these membrane proteins at a sufficient amount is a difficult task. Altogether, although much progress has been made in this field during the last years many questions remain open and are a challenge for future studies.

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CHAPTER II

Regulatory link between steryl ester formation and hydrolysis in the yeast *Saccharomyces cerevisiae*

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Published in Biochimica et Biophysica Acta (BBA) – Molecular and Cell Biology of Lipids

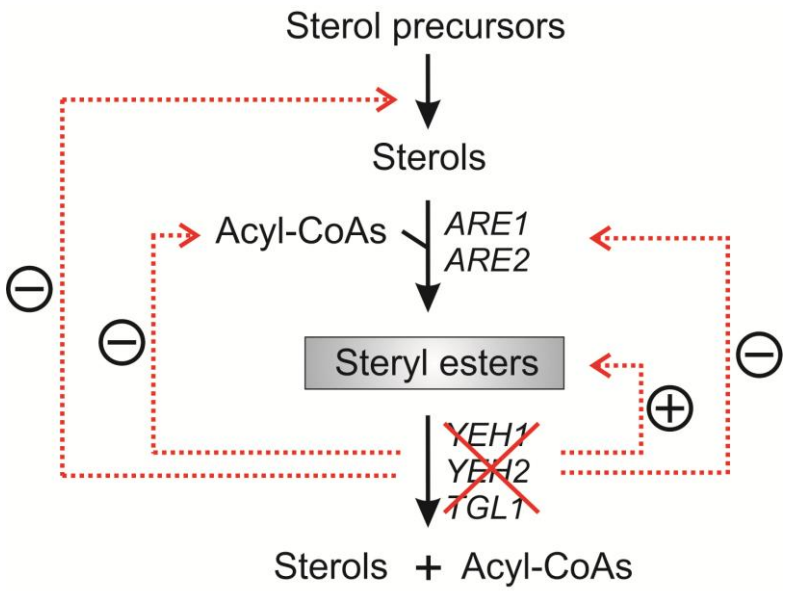
Volume 1851, Issue 7, Pages 977–986, 2015 July

doi: 10.1016/j.bbalip.2015.02.011

Abstract

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

Graphical abstract



Highlights

- Steryl esters and triacylglycerols are major storage lipids of the yeast.
- Steryl ester formation is feedback regulated by steryl ester hydrolysis.
- Defects in steryl ester hydrolysis affect the activity of steryl ester synthases.
- Lack of steryl ester hydrolysis reduces levels of sterols and fatty acids.

Key words: yeast, lipids, steryl ester(s), sterols, hydrolase, lipid droplets

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

1. Introduction

Sterol homeostasis is essential to maintain cellular membrane permeability and fluidity and to avoid harmful effects on membranes by an excess of free sterols. Yeast cells are unable to degrade sterols, and therefore mechanisms of sterol detoxification are needed. In *Saccharomyces cerevisiae*, three such mechanisms are known, namely (i) esterification of free sterols with fatty acids by the two acyltransferases Are1p and Are2p [1–3]; (ii) down regulation of sterol biosynthesis; and (iii) sterol acetylation by Aft2p and secretion by Pry-proteins [4–6]. Steryl esters (SE), formed by CoA-dependent acylation of sterols with fatty acids are stored in organelle-like structures called lipid droplets (LD) [7,8].

Are1p and Are2p are acyl-CoA:cholesterol acyltransferase related enzymes (ACAT), both located to the endoplasmic reticulum [1,2]. Although they share 49% sequence identity they have different substrate specificities. Are2p is regarded as the major acyltransferase accounting for more than 70% of total SE synthase activity and utilizes preferentially ergosterol as a substrate. Are1p was found to esterify mainly ergosterol precursors with a slight preference for lanosterol. Thus, Are1p may prevent harmful accumulation of sterol intermediates and form a depot of sterol intermediates which can rapidly re-enter the sterol biosynthetic pathway upon requirement [3]. A single deletion of *ARE2* decreases the cellular SE content to about 30% of wild type, whereas deletion of *ARE1* hardly affects the overall amount of SE. A mutant lacking both *ARE1* and *ARE2* completely lacks SE, but does not show any growth defects. It was demonstrated, however, that such a strain is synthetically lethal with *ARV1* (*ARE2* required for viability). Arv1p was found to play a role in sterol trafficking to the plasma membrane. Yeast cells lacking Arv1p showed altered intracellular sterol distribution and were defective in sterol uptake [9].

SE and triacylglycerols (TG) are the major non-polar lipids of yeast and preserve chemical energy and membrane building blocks. Under standard growth conditions TG are mainly synthesized by the diacylglycerol acyltransferase Dga1p and with a minor contribution by the second TG synthase Lro1p, both localized to the ER [10–13]. Additionally, the two SE synthases appear to contribute to TG synthesis, although to a small extent [14,15]. Both, TG and SE accumulate during the stationary growth phase of yeast cultures and are stored in LD. LD consist of a hydrophobic core of TG, surrounded by several shells of SE and a phospholipid monolayer with a distinct amount of proteins embedded [7,8,16]. Recently, the proteome of LD was investigated in more detail leading to a novel view of this organelle [8,17]. Nowadays, LD are no longer seen just as storage compartment but as dynamic

organelles which are also involved in the storage and degradation of protein aggregates or incorrectly folded proteins [18,19].

Non-polar lipids can be mobilized from LD upon requirement, for example during growth or starvation [8,16,20–22]. Mobilization of TG is carried out by TG lipases. In the yeast, three major TG lipases were identified, namely Tgl3p, Tgl4p and Tgl5p [23–25]. Recently, Ayr1p was also shown to serve as a further TG lipase although with minor lipolytic activity *in vivo* [26]. SE hydrolysis plays an important role in maintaining a balanced cellular sterol level [27]. In the yeast *S. cerevisiae*, the three SE hydrolases Yeh1p, Yeh2p and Tgl1p were identified [28,29], which are most likely the only SE hydrolases in this microorganism. Tgl1p and Yeh1p were localized to LD, whereas the highest activity of Yeh2p was detected in the plasma membrane. Tgl1p and Yeh1p were identified as paralogues of mammalian acid lipases, but their *in vitro* SE hydrolase activity is low. Yeh1p exhibits enhanced activity under heme deficiency mimicking anaerobiosis compared to standard growth conditions [28,30,31]. The three hydrolases have different substrate specificities. All three enzymes accept ergosteryl esters as substrate, but Tgl1p and Yeh2p show a slight preference for zymosteryl esters whereas Yeh1p efficiently hydrolyses fecosteryl esters [27]. In addition, Tgl1p was shown to degrade TG *in vitro*, although TG mobilization by this enzyme *in vivo* was minor [30].

SE synthesizing and degrading enzymes have been identified some years ago, but little is known about their regulation. Valachovic *et al*. (2002) showed that regulation of *ARE1* and *ARE2* is oxygen-dependent, and Are1p becomes especially important under hypoxic conditions [32]. Connerth *et al.* (2010) showed that Are2p is regulated by oleate which acts as a competitive inhibitor of the enzyme [33]. Moreover, it was demonstrated that deletion of *ARE1* and *ARE2* influenced sterol biosynthesis by changing the expression levels of Erg-genes, e.g. *ERG1* encoding squalene epoxidase, and *ERG3* encoding a C-5 sterol desaturase [34,35]. More evidence about regulatory aspects between catabolism and anabolism of non-polar lipids is still missing.

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

feedback regulation on Are1p and Are2p at the enzyme level and on the formation of sterols and fatty acids.

2. Materials and Methods

2.1. Yeast strains and culture conditions

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

Table 1 Yeast strains used throughout this study.

Strain	*Genotype*	*Source*
Wild type	BY4741 Mat a; *his3Δ1; leu2Δ0; met15Δ0; ura3Δ0*	Euroscarf
TM	BY4741; Δ*tgl1::kanMX4;* Δ*yeh1::kanMX4;* Δ*yeh2::kanMX4*	Wagner *et al*. (2009)
Δ*are1*Δ*are2*	BY4741; Δ*are1::kanMX4;* Δ*are2::kanMX4*	Wagner *et al*. (2009)
Δ*tgl1*	BY4741; Δ*tgl1::kanMX4*	Euroscarf
Δ*yeh1*	BY4741; Δ*yeh1::kanMX4*	Euroscarf
Δ*yeh2*	BY4741; Δ*yeh2::kanMX4*	Euroscarf
WT Are1-Myc	BY4741; *ARE1-13Myc::HIS3MX6*	This study
WT Are2-Myc	BY4741; *ARE2-13Myc::HIS3MX6*	This study
TM Are1-Myc	TM; *ARE1-13Myc::HIS3MX6*	This study
TM Are2-Myc	TM; *ARE2-13Myc::HIS3MX6*	This study
WT Erg2-Myc	BY4741; *ERG2-13Myc::HIS3MX6*	This study
TM Erg2-Myc	TM; *ERG2-13Myc::HIS3MX6*	This study

2.2. Genetic techniques

Chromosomal tagging of *ARE1*, *ARE2* and *ERG2* was performed according to the PCR-mediated method of Longtine *et al.* [36]. In brief, inserts of Are1-Myc, Are2-Myc and Erg2-Myc constructs were obtained by PCR from plasmid pFA6a-13Myc-HIS3MX6. Primers used for amplification of the respective DNA-fragments are listed in Table 2. Furthermore,

500 ng DNA were used for transformation of yeast strains employing the high-efficiency lithium acetate transformation protocol [37]. After transformation, cells were plated on minimal medium lacking histidine for selection and incubated for 2 to 3 days at 30°C. Positive transformants were verified by colony PCR of whole yeast cell extracts with primers listed in Table 2.

Table 2 Primers used throughout this study.

Primer	*Sequence (5' → 3')*
are1fw	TTGGTGTCTGTTCAGGGCCCAGTATCATTATGACGTTGTACCTGACCTTACGGAT CCCCGGGTTAATTAA
are1rev	TTGTATATCTATCAAGGGCTTGCGAGGGACACACGTGGTATGGTGGCAGTATCG ATGAATTCGAGCTCG
are2fw	TCGGTATCTGCATGGGACCAAGTGTCATGTGTACGTTGTACTTGACATTCCGGAT CCCCGGGTTAATTAA
are2rev	AAAATTTACTATAAAGATTTAATAGCTCCACAGAACAGTTGCACGATGCCATCG ATGAATTCGAGCTCG
Erg2F2	ACCTGACTGCCAGGGACATGGGTAAGAACTTGTTGCAAAACAAAAAGTTCCGGA TCCCCGGGTTAATTAA
Erg2S2	GTTATATTATAATGGACTACCGCATGACTGATTTCGTGAGGTCGGGCAGCATCGA TGAATTCGAGCTCG
are1fw_cPCR	GACCGCAGTTGTCCAACG
are2fw_cPCR	CAGAACCATAATCGGAAATGTTAT
Erg2confwd	GTACTCTTGATTTATACACTCTATAT
13Mycrev_cPCR	TATTTAGAAGTGGCGCGAATTCAC

2.3. Isolation and characterization of microsomes

Isolation of highly pure microsomes from cells grown to the early stationary phase was performed as described previously [38–40]. The protein concentration was analyzed by the method of Lowry *et al.* [41] using bovine serum albumin as a standard. Microsomal proteins were separated by SDS-PAGE using 12.5% polyacrylamide gels according to the method of Laemmli [42]. Western Blot analysis was performed as described by Haid and Suissa [43]. Proteins were detected using mouse or rabbit antisera, respectively, as primary antibody directed against Myc-tag and Wbp1p (ER-marker). Primary antibodies were detected with peroxidase-conjugated goat anti-mouse or anti-rabbit IgG, respectively, as second antibody and SuperSignal® West Pico Chemiluminescent substrate solution.

2.4. Western blot analysis

To analyze the protein stability of Are1p-Myc and Are2p-Myc, Western blot analysis was performed with total cell extracts from wild type and TM cultivated for different time periods in the presence of 100 µg/mL cycloheximide added to cells grown to the mid-logarithmic phase. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as loading control. To quantify cellular amounts of Erg2p-Myc and Erg6p, Western blot analysis was carried out with cell homogenates using mouse anti-Myc antibody or rabbit anti-Erg6p antibody, respectively, as primary antibodies. Detection of primary antibodies was performed as described above.

2.5. Lipid extraction and analysis

Non-polar lipid analysis was performed from total yeast cells. Homogenates were prepared from cells grown to the stationary phase. Cells were suspended in breaking buffer (50 mM TrisHCl, pH 7.4, 150 mM NaCl) and disintegrated by vigorous shaking in the presence of glass beads for 10 min at 4°C. Cell debris were removed by centrifugation at 5,000 x g for 5 min, and the supernatant was used for protein determination and lipid extraction using chloroform/methanol (2:1; v/v) as solvent [44]. For the quantification of non-polar lipids, lipid extracts were applied to Silica Gel 60 plates, and chromatograms were developed by a two-step developing system using light petroleum/diethyl ether/acetic acid (35:15:1 per volume) as the first solvent system for two thirds of the plate. After drying plates, chromatograms were further developed to the top of the plate using light petroleum/diethyl ether (49:1; v/v) as the second solvent system. Non-polar lipids, including TG, SE and free sterols were visualized by post-chromatographic charring of bands after dipping plates in a solution consisting of 0.63 g of $MnCl_2$ x 4 H_2O, 60 mL water, 60 mL methanol and 4 mL concentrated sulfuric acid. Plates were then heated at 105°C for 40 min. Bands were quantified by densitometric scanning (CAMAG TLC SCANNER 3) at 400 nm by comparing to authentic standards containing defined amounts of the respective lipids. For quantification of total phospholipids, a lipid extract from total cells (800 µg protein) was analyzed by the method of Broekhuyse [45].

Total sterols, including esterified and non-esterified forms of sterols as well as sterol intermediates, were analyzed as described previously [46]. In brief, homogenate corresponding to 1 mg protein was incubated for 2 h at 90°C with 600 µL methanol, 400 µL 0.5% pyrogallol dissolved in methanol, 400 µL 60% aqueous KOH, and 10 µg cholesterol

dissolved in ethanol as internal standard. After cooling to room temperature, lipids were extracted three times with 1 mL n-heptane, and combined extracts were dried under a stream of nitrogen. Extracted sterols were dissolved in 10 µL pyridine and derivatized with N,O-bis(trimethylsilyl)trifluoroacetamid (Sigma). After an incubation time of 15 min samples were diluted with 80 µL ethyl acetate. GLC/MS was performed on a Hewlett-Packard HP 5890 Series II gas chromatograph (Palo Alto, CA), equipped with an HP 5972 mass selective detector, and HP 5-MS column (cross-linked with 5% phenyl methyl siloxane; dimensions 30 m x 0.25 mm x 0.25 µm film thickness). Aliquots of 1 µL were injected in the splitless mode at 270°C injection temperature with helium as a carrier gas at a flow rate of 0.9 mL/min in constant flow mode. The temperature program used was 1 min at 100°C, 10°C/min to 250°C, and 3°C/min to 300°C. Mass spectra were acquired in scan mode (scan range 200-259 amu) with 3.27 scans per second. Sterols derived from esterified and non-esterified forms were identified by mass fragmentation patterns.

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

2.6. Quantitative Real Time-PCR

Total RNA isolation from cells grown to the mid-logarithmic phase was performed as described by the manufacturer using the RNeasy kit from Qiagen. After DNaseI digestion, quantitative Real Time PCR was performed using SuperScript III Platinum SYBR Green One-Step qRT-PCR Kit (Invitrogen) according to the manufacturer's instructions. Reactions were performed in sealed MicroAmp Optical 96-Well Reaction Plates, and amplification was measured using an ABI 7500 instrument (Applied Biosystems). Samples were quantified using the ΔΔCt method described by Livak and Schmittgen [47]. Primers used for Real Time-PCR are listed in Table 3.

Table 3 Primers used for qRT-PCR.

Primer	*Sequence (5' → 3')*
Are1_RTFw	CTTCACTGTTCTGTACATGCTCACGTTTTACATG
Are1_RTRev	CGAAACGCAATTCCACCAGTCGCCGTAG
Are2_RTFw	GATGTATCCTGTAGCAATGAGAGCATTGGCTGTG
Are2_RTRev	CTGGGACGATATCAACGAGCAATCCAAC
Erg11-RTFw	GTTCCGTAAGGTTATGAAAGATATGCACGTTC
Erg11-RTRev	GTATTCGTCTCTTAAATGAGTGTAACCTGGAG
Erg2-RTFw	CAGCATGCCAGGTGGTTCCTTTG
Erg2-RTRev	GTGTATAAATCAAGAGTACTGGAGAAAGTGTC
Erg6-RTFw	CGAAGTCCTCGTTAGCGAAGAC
Erg6-RTRev	GTGGCCAAATTAGCTAAGTTTTGAACGTAC
ACT1_RTFw	CCAGCCTTCTACGTTTCCATCCAAG
ACT1_RTRev	GACGTGAGTAACACCATCACCGGA

2.7. Enzymatic analysis

Acyl-CoA:ergosterol acyltransferase activity was measured using 70 µg protein from microsomal fractions, or 250 µg protein from homogenate as enzyme source in a final volume of 200 µL. Homogenate was prepared after digesting yeast cell walls using zymolyase as described previously [38–40]. The assay mixture contained 100 mM potassium phosphate buffer, pH 7.4, 25 µM ergosterol, 0.5 mM CHAPS, 10 mM DTT, 1.7 µM [^{14}C]oleoyl-CoA (58.6 mCi/mmol; PerkinElmer Life Sciences) and 50 µM unlabeled oleoyl-CoA (Sigma). Samples were incubated at 30°C for 15 min in a water bath. The reaction was stopped by adding 3 mL chloroform/methanol (2:1, v/v), and lipids were extracted by vortexing. Chromatograms were developed by the two step system described above. After reversible staining with iodine vapor bands of SE were scraped off the plates, and radioactivity was measured by liquid scintillation counting using 8 mL LSC Safety Cocktail (Baker, Deventer, The Netherlands) containing 5% water as scintillation cocktail. For a more detailed enzymatic analysis, acyl-CoA:ergosterol acyltransferase assays were performed with different concentrations of exogenously added ergosterol or oleoyl-CoA, respectively; and different amounts of the enzyme source as will be outlined in the Results section. When varying the ergosterol concentration in a range from 1 µM to 100 µM, 1.7 µM [^{14}C]oleoyl-CoA and 50 µM unlabeled oleoyl-CoA were used. For variation of the oleoyl-CoA in a range of 0.5 µM to 50 µM, ergosterol was used at a concentration of 25 µM.

2.8. *In vivo* labeling of lipids

To analyze the incorporation of fatty acids and acetate into complex lipids *in vivo*, wild type and *Δtgl1Δyeh1Δyeh2* were inoculated to an A_{600} of 0.1 in 500 mL YPD and grown to the early stationary phase. An aliquot of 20 mg cell wet weight (CWW) in a total volume of 10 mL YPD was pre-incubated with stirring for 15 min at 30°C in a water bath. Cells were then incubated for 20 min at 30°C in the presence of 10 µL [$^{1\text{-}14}$C]oleic acid (50 mCi/mmol; PerkinElmer Life Sciences) or 20 µL [$^{1,2\text{-}14}$C]-acetate (45-60 mCi/mmol; PerkinElmer Life Sciences), respectively. Aliquots of 2 mL were harvested by centrifugation after addition of 1 mL 2% BSA. The cell pellet was washed once with pre-cooled BSA solution (0.1%) and twice with cold distilled water. Then, cells were suspended in ice-cold water and disintegrated for 10 min at 4°C by vigorous shaking in the presence of glass beads. Lipids were extracted and separated by TLC as described above. Bands of TG, SE and phospholipids were scraped off the plates, and radioactivity was measured as described above.

3. Results

3.1. Lipid analysis of strains bearing defects in steryl ester metabolism

Yeh1p, Yeh2p and Tgl1p are the major SE hydrolyzing enzymes of the yeast *S. cerevisiae*, whereas synthesis of SE is catalyzed by the two acyltransferases Are1p and Are2p [1–3,28,29]. To investigate the interplay between SE hydrolysis and SE formation, we first analyzed the amount of SE in strains bearing defects in SE metabolism (Fig. 1A). We compared wild type (WT) to strains lacking either one or all three SE hydrolases (TM; Δ*tgl1Δyeh1Δyeh2*), and a strain deficient in the two acyltransferase Are1p and Are2p. In the stationary growth phase, when cells accumulated non-polar lipids, the TM showed about 30% more SE than WT. Interestingly, the *Δtgl1* strain accumulated SE at the same amount as the TM, whereas *Δyeh1* and *Δyeh2* single mutants showed SE levels similar to WT. These data confirmed previous studies of our laboratory [27] using YPD or MMGal (minimal media with 2% galactose) as cultivation media. The TM as well as a strain lacking the two acyltransferases Are1p and Are2p showed also increased amounts of TG (Fig. 1B). This metabolic link between TG and SE synthesis and mobilization confirmed recent findings by Schmidt *et al.* [48].

To get a broader view of the regulatory network of SE metabolism, we also analyzed phospholipids of the TM, the double deletion strain *Δare1Δare2* and WT (Fig. 1C). The total

amount of phospholipids was not altered in the TM, whereas the *Δare1Δare2* double mutation led to elevated amounts of phospholipids. The latter result confirmed a link of non-polar and phospholipid metabolism as also shown previously [49,50].

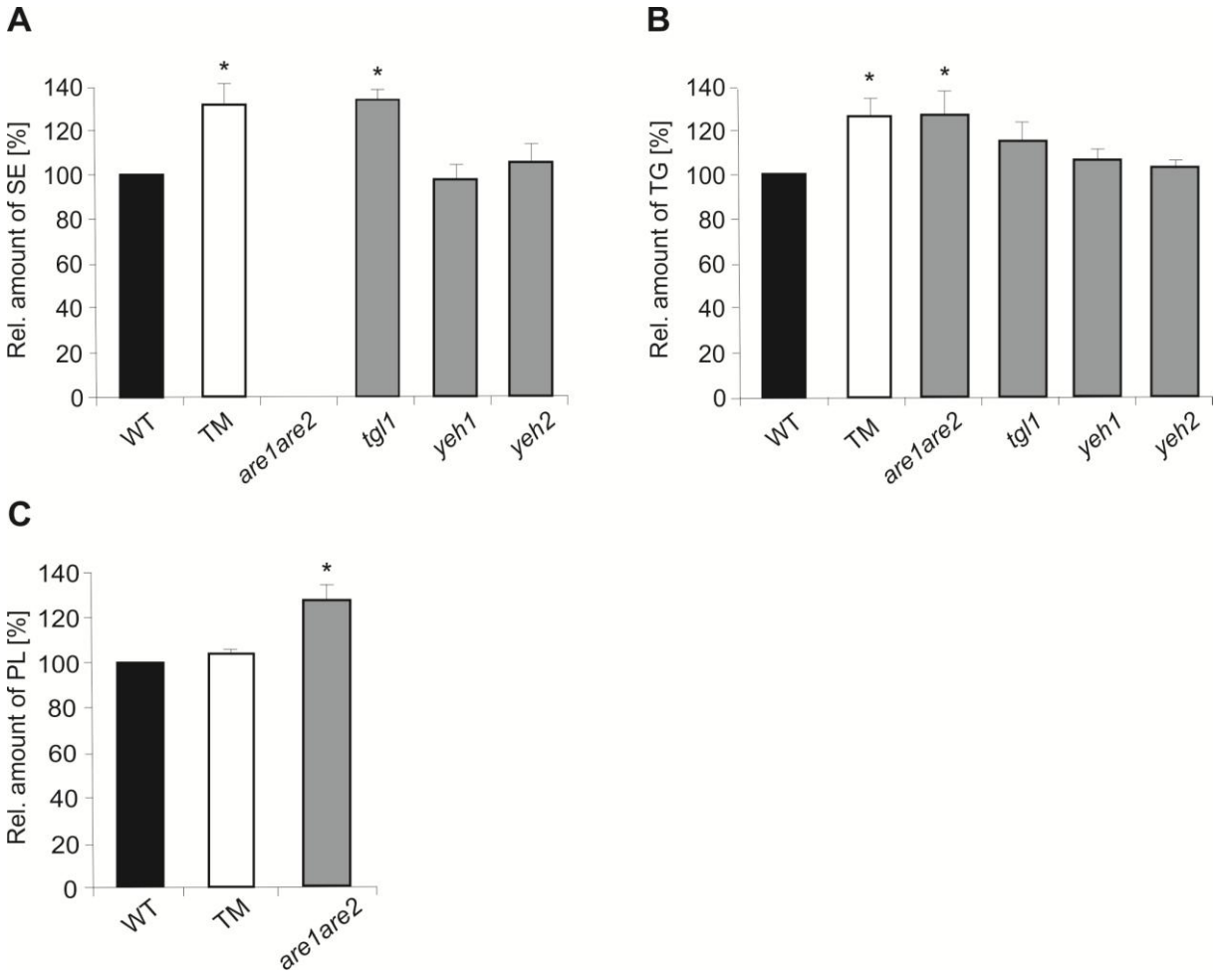


Fig. 1. Lipid analysis of yeast strains bearing mutations in steryl ester metabolism.
Data of wild type (WT), *Δtgl1Δyeh1Δyeh2* (TM), *Δare1Δare2*, *Δtgl1*, *Δyeh1*, and *Δyeh2* are shown. (A) Relative amount of steryl esters (SE). (B) Relative amount of triacylglycerols (TG). (C) Relative amount of phospholipids (PL). The amounts of SE, TG and PL from WT were set at 100%. Data are mean values of at least three independent experiments performed in duplicate with the respective standard deviations as indicated. Values indicated by * correspond to $p < 0.05$ and were defined significant.

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

To shed more light on the synthesis of SE when their hydrolysis is blocked we performed *in vivo* labeling of lipids in wild type and *Δtgl1Δyeh1Δyeh2*. We followed the incorporation of [^{14}C]oleic acid into the main lipid classes in cultures which have reached the stationary growth phase. Fig. 2A shows the incorporation of [^{14}C]oleic acid into phospholipids, TG and SE. The total incorporation of [^{14}C]oleic acid into lipids from the TM (1,000 ± 80 cpm/mg CWW) was similar to WT (1,100 ± 30 cpm/mg CWW). In wild type, more than half of the label was found in phospholipids, whereas the other half was distributed among non-polar lipids. SE and TG were formed at nearly equal amounts. The TM deficient in all three SE hydrolases incorporated the same amount of [^{14}C]oleic acid into phospholipids as WT, but showed a significant shift of the label from SE to TG. This result indicated that TG formation was enhanced, whereas the formation of SE was decreased in cells lacking SE hydrolyzing enzymes. Thus, a feedback regulation on Are1p and Are2p occurred when the hydrolysis of SE was blocked.

A similar experiment was performed with [^{14}C]acetate as lipid precursor to get some additional information about fatty acid biosynthesis in the TM. Fig. 2B shows that also [^{14}C]acetate incorporation into SE was reduced in the TM. Most interestingly, the overall incorporation of [^{14}C]acetate into lipids was decreased in the TM (4,700 ± 120 cpm/mg CWW) compared to WT (6,700 ± 450 cpm/mg CWW) suggesting a reduced overall lipid formation in the mutant strain.

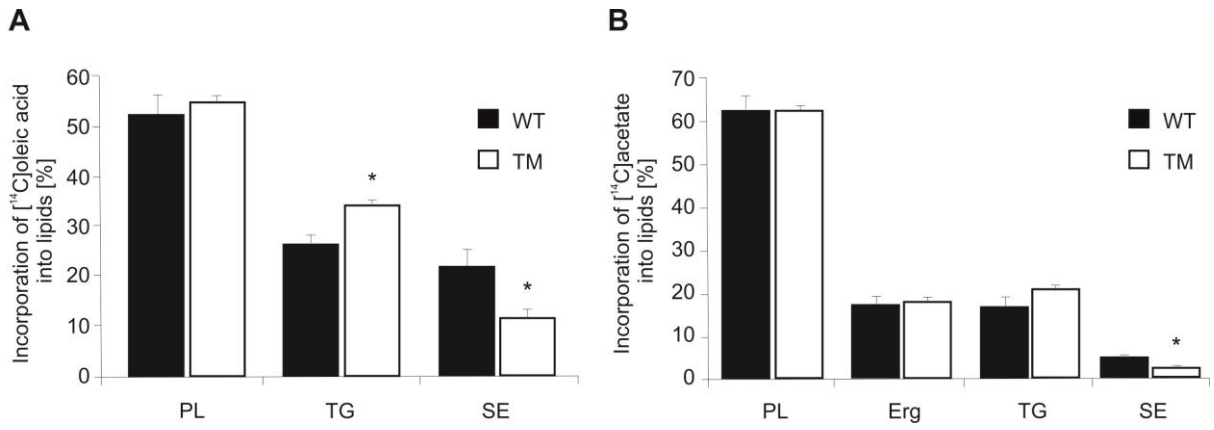


Fig. 2. *In vivo* labeling of lipids.

In vivo labeling with [^{14}C]oleic acid (A), and [^{14}C]acetate (B) of lipids from cells lacking the three steryl ester hydrolase (TM) compared to wild type (WT) is shown. Relative

incorporation of labels into phospholipids (PL), triacylglycerols (TG), steryl esters (SE) and ergosterol (Erg), respectively, was analyzed. The total incorporation of [^{14}C]oleic acid into TM was 1,000 ± 80 cpm/mg CWW, and into WT 1,100 ± 30 cpm/mg CWW. In contrast, the total incorporation of [^{14}C]acetate into lipids of TM and WT was different. In the TM 4,700 ± 120 cpm/mg CWW, and in WT 6,700 ± 450 cpm/mg CWW were incorporated. Data are mean values of two independent biological experiments analyzed in duplicate with the respective standard deviations as indicated. Values indicated by * correspond to $p < 0.05$ and were defined significant.

To substantiate a possible feedback control to fatty acid and sterol synthesis we quantified the amounts of sterols and fatty acids in wild type and TM. Sterol quantification by GLC/MS (Table 4) showed that deletion of the three SE hydrolases led to a decreased level of total cellular sterols to about 65% of WT. Noteworthy, these values included amounts of free sterols and sterols in the esterified form of SE and thus represented the overall sterol production in the respective strains.

Table 4 Sterol analysis by GLC/MS from wild type (WT) and Δ*tgl1Δyeh1Δyeh2* (TM). Data are mean values from three independent experiments performed in duplicate.

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

Quantification of free sterols (non-esterified form) can be seen in Fig. 3A. The reduced amount of free sterols in the TM to ~70% of wild type could be due either to insufficient sterol biosynthesis in the TM or to enhanced secretion of sterols in their acetylated forms. To exclude the latter possibility, lipids from the cell pellet and the media of cells grown to the stationary phase were analyzed. In both wild type and TM the amount of

secreted steryl acetate was marginal, and there was no difference between the two strains (data not shown). These results suggested that changes in SE hydrolysis rather influenced total sterol biosynthesis. Interestingly, the lack of SE hydrolases did not only change the total sterol content, but also the sterol pattern (Table 4). The amount of the end product of the sterol biosynthetic pathway, ergosterol, was markedly decreased in the TM, whereas some precursors such as zymosterol and lanosterol accumulated. These results suggested that certain steps of the ergosterol biosynthetic pathway may be constricted in the TM.

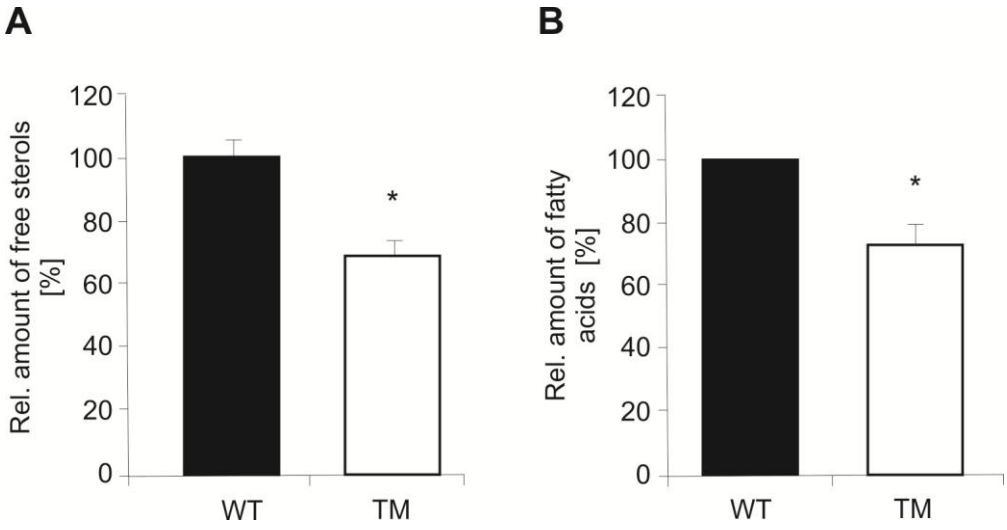


Fig. 3. Quantification of free sterols and fatty acids.

Relative amounts of (A) free sterols and (B) fatty acids in wild type (WT) and *Δtgl1Δyeh1Δyeh2* (TM) were analyzed. Free sterols were quantified by thin layer chromatography, and fatty acids were determined by GLC. Data are mean values of two independent experiments performed in duplicate with the respective standard deviations as indicated. Values indicated by * correspond to $p < 0.05$ and were defined significant.

Indeed, expression levels of some *ERG* genes, e.g. *ERG2* and *ERG6*, were slightly decreased (Fig. 4A), although the respective protein levels were more or less the same in the TM and WT (Figs 4B and 4C). Labeling experiments with [^{14}C]acetate described above suggested that the formation of the second substrate of SE formation, the fatty acids, was reduced in the TM. Thus, it was not surprising that the steady state level of total fatty acids in the TM was lower than in WT (Fig. 3B). This result fostered our idea that a block in SE hydrolysis also led to some substrate limitation for SE synthases.

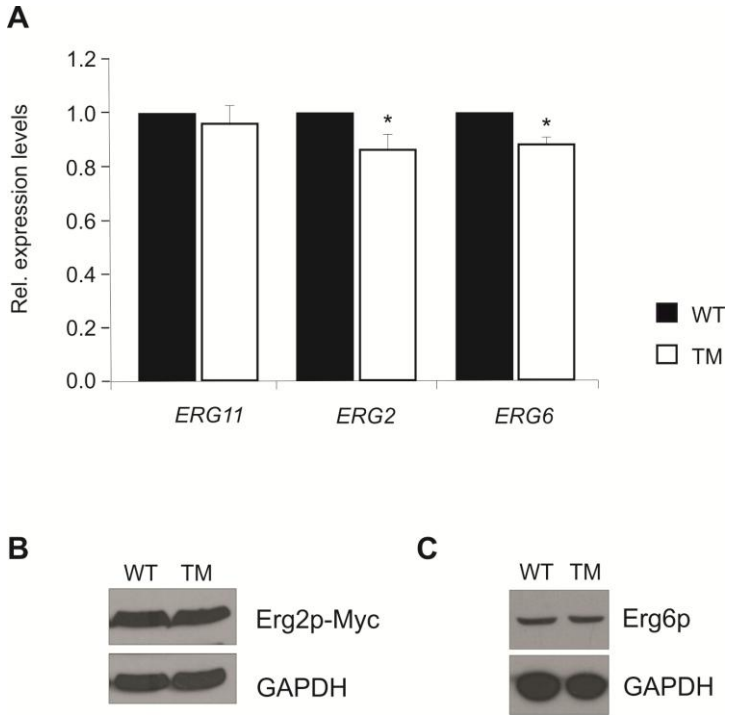


Fig. 4. Gene expression and protein levels of Erg11p, Erg2p and Erg6p.

(A) Relative gene expression of *ERG11*, *ERG2* and *ERG6* in wild type (WT) (black bar) and TM (white bar) was measured by RT-PCR from cells grown to the mid-exponential growth phase. Wild type was set at 1. Data are mean values from two independent experiments with the respective deviation. (B) Protein analyses of Erg6p and Erg2p-Myc from homogenate of WT and TM are shown. Protein amounts corresponding to 1 A_{600} unit of cells were applied to the SDS-gel. Western blot analyses are representative of at least two independent experiments. GAPDH was used as loading control. Values indicated by * correspond to $p < 0.05$ and were defined significant.

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

Alterations in SE amounts as a consequence of defects in SE hydrolysis raised the question as to the regulation of SE synthesis. The major question addressed in the present study was how Are1p and Are2p behave when SE hydrolysis was blocked. We hypothesized that the synthesis of SE was down regulated in a yeast strain lacking all three SE hydrolases. This feedback inhibition could either be on the gene expression level, the protein level or the

enzymatic level of the two SE synthesizing enzymes Are1p and Are2p. First, we investigated the expression levels of *ARE1* and *ARE2* in WT and in the TM by Real-Time PCR. As can be seen from Figs 5A and 5B, the gene expression of *ARE1* and *ARE2* was not significantly altered in the *Δtgl1Δyeh1Δyeh2* mutant. This result excluded a regulation at the expression level. Next, we investigated whether the protein levels of the two acyltransferases were affected. Western Blot analysis of tagged versions of these enzymes performed with microsomal fractions indicated no changes in the steady state levels of Are1p and Are2p (Figs 5C and 5D). As stability of the two enzymes was another possibility of regulation, we also tested amounts of Are1p-Myc and Are2p-Myc from total cell extracts of wild type and TM grown for different time periods after addition of 100 µg/mL cycloheximide to cells grown to the mid-logarithmic phase. Western blot analysis revealed no marked differences between TM and WT (Fig. 5E and F).

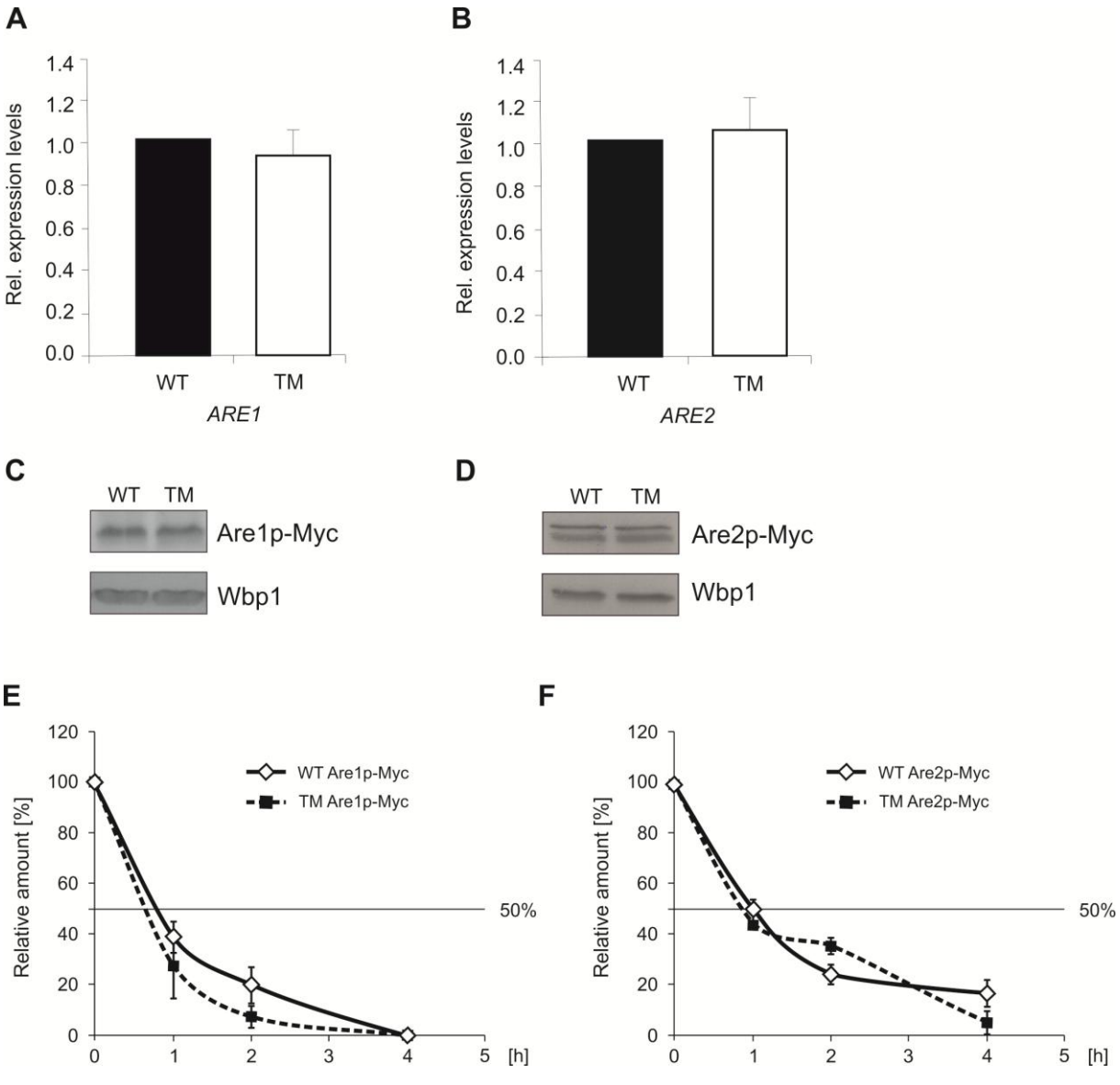


Fig. 5. Gene expression, protein levels and protein stability of Are1p and Are2p.
Relative gene expression of *ARE1* (A) and *ARE2* (B) in wild type (WT) (black bar) and TM (white bar) was measured by RT-PCR from cells grown to the mid-exponential growth phase. Wild type was set at 1. Data are mean values from three independent experiments with the respective deviation. Are1p-Myc (C) and Are2p-Myc (D) from microsomal fractions of WT and TM grown to the early stationary phase were quantified by Western blot analyses in at least two independent experiments. For SDS-PAGE 40 µg protein per lane was loaded. The appearance of two bands is most likely due to degradation of Are2p-Myc. However, both bands occurred at the same intensity in WT and TM. Wbp1p (ER marker) was used as loading control. (E) Stability of Are1p-Myc and (F) Are2p-Myc in wild type and TM was measured by Western blotting. Protein amounts were quantified using the ImageJ program. Are1p-Myc and Are2p-Myc were quantified from total cell extracts from wild type and TM grown for time periods as indicated after addition of 100 µg/mL cycloheximide to cells grown to the mid-logarithmic phase. GAPDH was used as loading control. Western blot analyses were performed in biological duplicates.

Results obtained by Real-Time PCR and Western Blot analysis suggested that a block of SE hydrolysis most likely affected the activity of SE synthesizing enzymes at the enzymatic level. To test this hypothesis we performed *in vitro* enzyme assays of Are1p and Are2p. Isolated ER fractions from wild type and *Δtgl1Δyeh1Δyeh2* were used as enzyme sources. As can be seen from Figs 6A and 6B, the acyltransferase activity was dramatically decreased in both homogenate and microsomal fractions from the TM. To exclude an influence of assay conditions on the obtained results, we varied the substrate and the detergent in the acyltransferase assays. However, use of palmitoyl-CoA instead of oleoyl-CoA as substrate, as well as replacement of CHAPS by Tyloxapol did not change the effect of the triple mutation (data not shown). To investigate the contribution of individual SE hydrolases to the reduced enzyme activities of the two acyltransferases we also performed *in vitro* assays with single deletion strains. Fig. 5C shows that each of the three SE hydrolases contributed partially to the reduced activity of Are1p/Are2p with the most pronounced effect of Tgl1p. Enzyme assays with tagged variants of Are1p and Are2p which were used for expression studies described above yielded identical results (data not shown), indicating that the tag did not influence the activity of the enzymes.

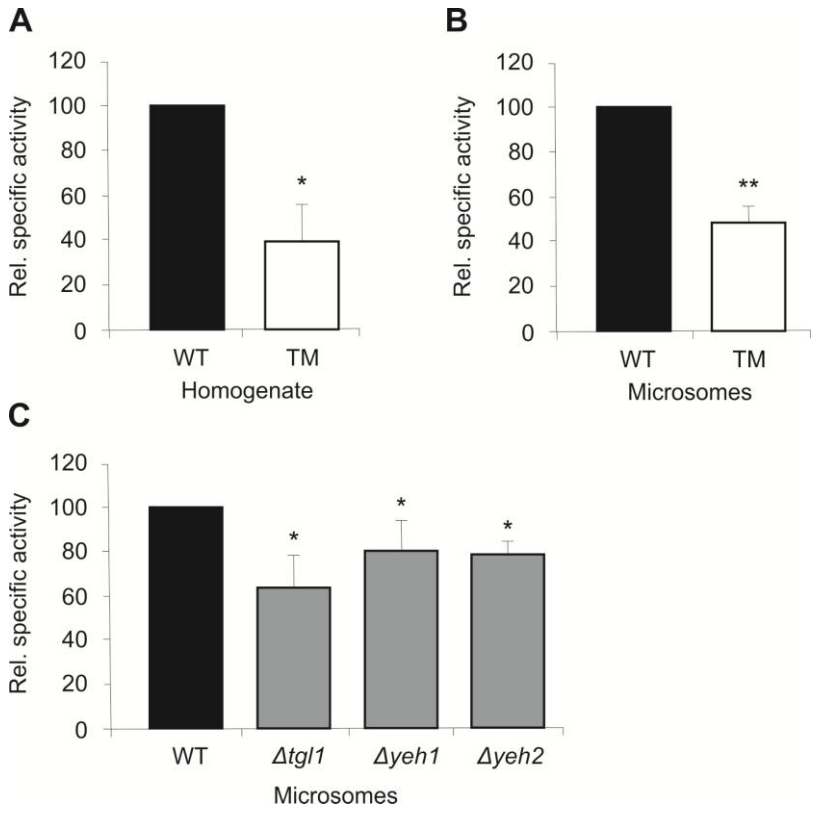
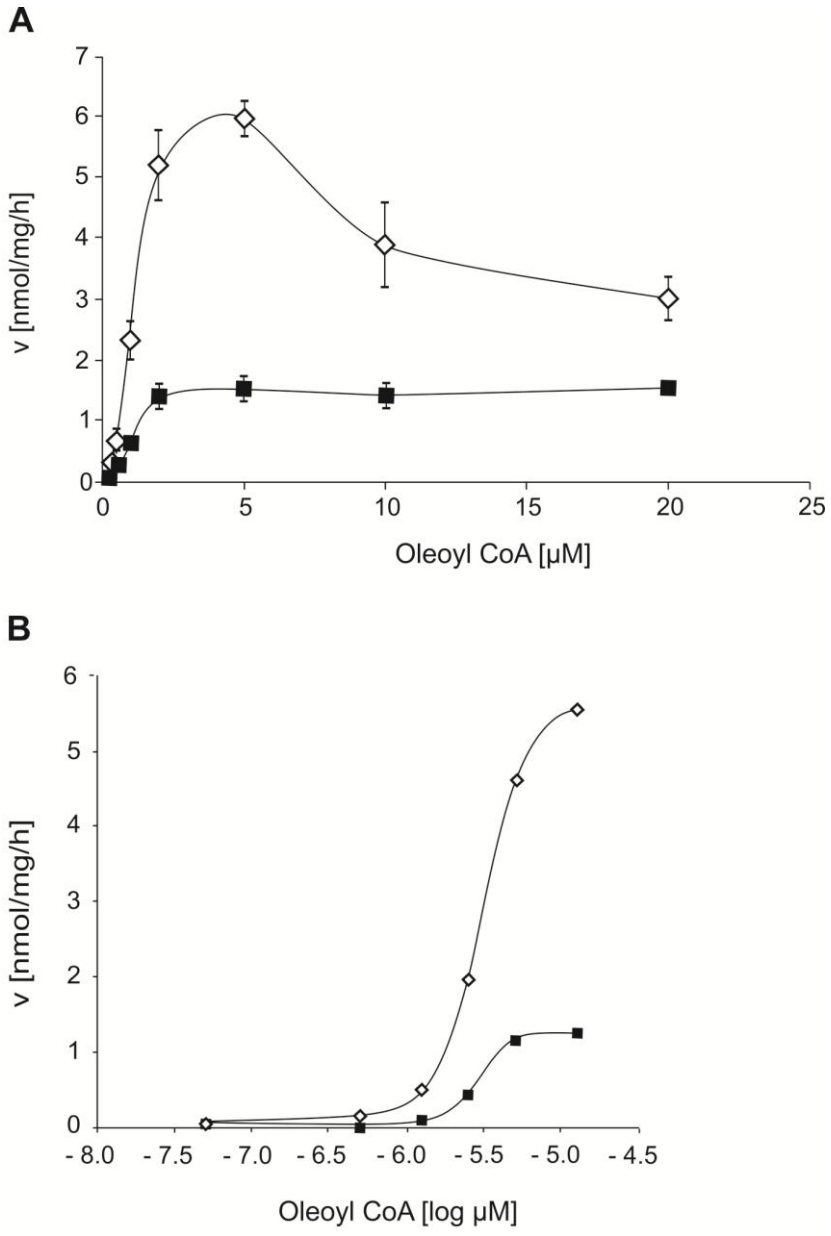


Fig. 6. Acyl-CoA:ergosterol acyltransferase activity of Are1p/Are2p in strains bearing a defect in SE hydrolysis.

(A) Homogenate was used as enzyme source. Specific activity of acyl-CoA:ergosterol acyltransferase activity from wild type (WT) (1100 pmol SE/mg protein/h) was set at 100% and compared to *Δtgl1Δyeh1Δyeh2* (TM). (B) Microsomal fractions were used as enzyme source. Specific activity of acyl-CoA:ergosterol acyltransferase activity from WT (8,300 pmol SE/mg protein/h) was set at 100% and compared to *Δtgl1Δyeh1Δyeh2* (TM). (C) Microsomes from single deletion mutants were compared with microsomes from WT. Specific activity of acyl-CoA:ergosterol acyltransferase activity of wild type (WT) (8,300 pmol SE/mg protein/h) was set at 100%. Data shown are mean values of at least three independent experiments performed in duplicate with the respective standard deviations as indicated. Values indicated by * correspond to $p < 0.05$, and ** to $p < 0.01$ and were defined significant.

To further investigate reasons of the reduced activities of the two SE synthases Are1p and Are2p in the TM, enzyme kinetics were measured. In these assays, the amounts of the substrates oleoyl-CoA (Figs 7A and 7B) and ergosterol (Fig. 7C) were varied. As can be seen from these data, the enzymatic properties of steryl ester synthases varied dramatically in wild type and TM. Whereas v_{max} showed a big difference, the K_m values calculated using the

GraphPad Prism software (Fig. 7B) were similar for wild type and TM, namely 3.1 µM and 2.9 µM, respectively. Interestingly, a strong substrate inhibition of the reaction by oleoyl-CoA (see Figs 7A and 7B) was detected with wild type, whereas such an effect was not seen with the TM. Variation of the amounts of ergosterol as a substrate confirmed the different v_{max} of SE synthesis in wild type and TM. In the case of ergosterol, however, the endogenous amount of the substrate in the enzyme samples (microsomes) was already at a saturation level and did not allow further analysis of enzymatic parameters. Results described above also demonstrated that substrate limitation was not an issue for *in vitro* assays.



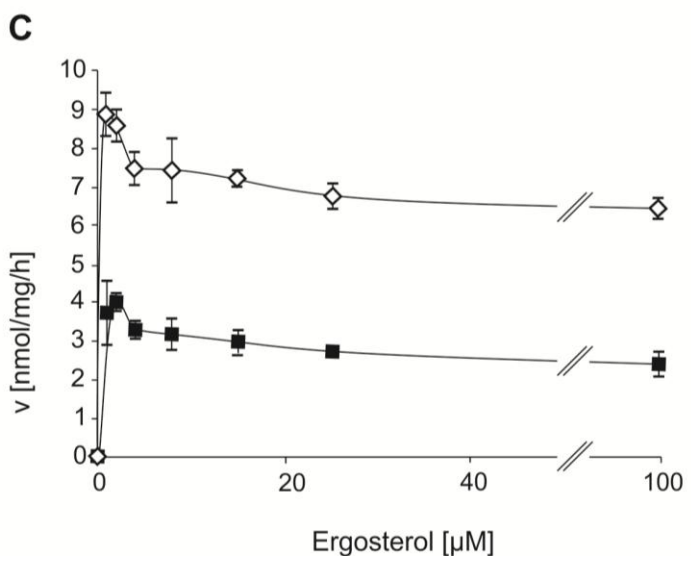


Fig. 7. Enzyme kinetics of steryl ester synthase.

SE formation in vitro was measured in an oleoyl-CoA (A, B) and ergosterol (C) dependent manner. Assays were performed as described in the Methods section. Oleoyl-CoA concentrations between 0.5 µM and 50 µM were used (A, B). Exogenous ergosterol concentrations were varied between 1 µM and 100 µM (C). GraphPad Prism software was used to plot reaction velocity values against the logarithm of oleoyl-CoA concentration (B). ◊ wild type; ■ *Δtgl1Δyeh1Δyeh2* triple mutant

The question remained whether or not components present in microsomal samples from the TM used as enzyme source might cause an inhibitory effect on SE synthesis. To address this problem a mixing experiment of microsomes from wild type and TM as enzyme source was performed. As references, assays with increasing amounts of wild type or TM microsomes, respectively, were carried out. As can be seen from Fig. 8, wild type and TM showed the expected behavior, namely strong reduction of SE synthesis activity in the TM. However, when TM microsomes were added to wild type microsomes (35 µg protein), an additive behavior was observed. As no stronger reduction of the activity was seen we concluded that intrinsic properties of the enzymes from the different strains were the reason for the observed effect. We also analyzed phospholipids from wild type and TM microsomes to test whether these components may have influenced the enzyme behavior. As no changes were observed in wild type and *Δtgl1Δyeh1Δyeh2* (data not shown) this possibility was also excluded.

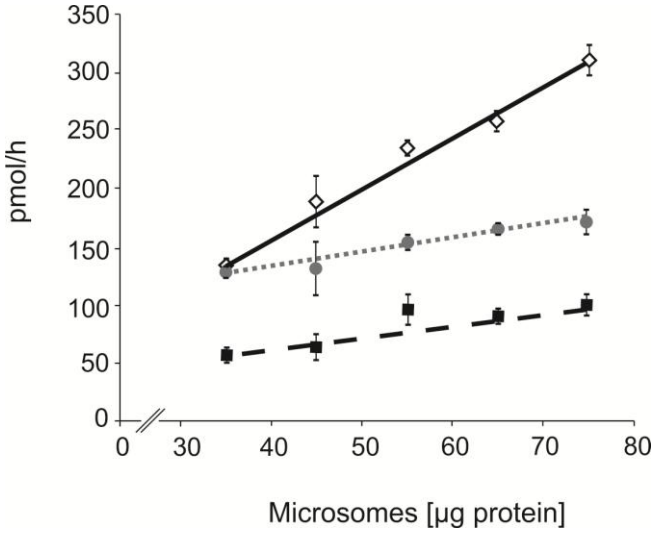


Fig. 8. Steryl ester synthesis *in vitro* in a mixed assay with microsomes from wild type and *Δtgl1Δyeh1Δyeh2*

Acyl-CoA:ergosterol acyltransferase assays were performed with different amounts of proteins from microsomes as enzyme source. Starting conditions were microsomes (35 µg proteins) from wild type (◊); and the *Δtgl1Δyeh1Δyeh2* triple mutant (TM; ■). Increasing amounts of wild type and TM microsomes were added as indicated. In the mixing experiment (●) increasing amounts of TM microsomes were added to wild type microsomes (35 µg proteins).

4. Discussion

Storage of the non-polar lipids SE and TG in lipid droplets is conserved from yeast to man [51]. Although the functional characterizations as well as the substrate specificities of steps involved in non-polar lipid metabolism were studied in some detail, little is known about the regulation of this process [32–34]. Here, we provide some insight into regulatory aspects of the SE metabolic network in *S. cerevisiae*. We have chosen to study effects on the formation of SE in cells deleted of *TGL1*, *YEH1* and *YEH2* which encode the three known SE hydrolases of the yeast. We analyzed transcriptional and translational control, protein stability as well as substrate availability as possible mechanisms regulating the activity of the two SE synthesizing enzymes Are1p and Are2p. In addition, we provide evidence for a metabolic link between SE metabolism and the biosynthesis of sterols and fatty acids.

In this study, we show that regulation of sterol acyltransferase activity in a TM lacking *TGL1*, *YEH1* and *YEH2* does not occur on the gene expression level of *ARE1* and *ARE2* (see Figs 5A and 5B). Also protein levels and protein stability of Are1p and Are2p were not significantly altered in the TM compared to wild type (see Figs 5C, 5D, 5E and 5F). Nevertheless, lipid formation analyzed by *in vivo* labeling experiments showed that a block of SE hydrolysis led to reduced SE formation whereas TG formation was enhanced. *In vitro* enzyme assays revealed that the activity of Are1p and Are2p was markedly reduced in the TM (see Fig. 2 and Fig. 6). More detailed enzymatic analyses (see Fig. 7) demonstrated that SE formation in wild type and TM obeyed to different kinetics. The clearly reduced maximum activity of the reaction and an apparent loss of substrate inhibition in the TM are the major characteristics of these assays. Although effects observed *in vivo* and *in vitro* were similar it has to be mentioned that *in vivo* activities cannot always be strictly and numerically correlated to *in vitro* results. Also the influence of the reduced amount of substrate formation (see Table 4) has to be interpreted with caution as we cannot deduce from these results the local availability of acyl-CoA and sterol to Are1p and Are2p.

Based on these results we concluded that deletions of *TGL1*, *YEH1* and *YEH2* cause multiple effects of feedback regulation. The reduced formation of ergosterol in the TM deserved our special attention. Previous studies have already provided evidence for such a related feedback mechanism of SE metabolism [34,35]. Sorger *et al.* [34] reported that the amount of the squalene epoxidase Erg1p was decreased in the Δ*are1*Δ*are2* double mutant. This effect was caused by low stability of Erg1p in this strain. Arthington-Skaggs *et al*. [35] found that the expression of *ERG3* was down-regulated in the absence of sterol esterification. These authors provided the first evidence for direct transcriptional regulation of a sterol biosynthetic gene in response to sterol esterification. Our data presented here revealed that the expression of *ERG2* and *ERG6* was slightly decreased in the TM compromised in SE hydrolysis. A link between *ERG6* expression and SE formation has been shown previously by McCammon *et al.* [53]. Also the feedback inhibition to fatty acid formation by the Δ*tgl1*Δ*yeh1*Δ*yeh2* triple mutations seems logical as the cellular amount of fatty acids required is decreased in the TM due the block of SE turnover. Effects of fatty acid availability on the formation of non-polar lipids were recently discussed by Mora *et al.* [52].

The enhanced formation of TG in the TM indicates a link between TG formation and SE metabolism. Evidence of a “cross-talk” between SE and TG metabolism was recently also

observed in another study from our lab [48]. We showed that yeast strains defective in the three major TG lipases Tgl3p, Tgl4p and Tgl5p produced more SE than wild type. Although a balanced distribution between TG and SE seems to be a way to get rid of the excess of fatty acids, the question as to the specific regulation within the non-polar lipid metabolic network remains. The fact that fatty acids were not randomly distributed over all lipid classes was demonstrated by the finding that phospholipids in the Δ*tgl1*Δ*yeh1*Δ*yeh2* triple mutant were not overproduced. A new facet of SE and TG metabolism was the identification of novel hydrolases and lipases which appear to come to the fore when the main TG lipolytic activities are blocked [26,54,55]. It has to be considered that such enzymes may compensate for deletions of the major hydrolytic enzymes. Another interesting aspect which has to be taken into account is the subcellular localization of the different SE hydrolases. Although the natural substrates of these enzymes, SE are stored in LD, the major SE hydrolase Yeh2p was localized to the cell periphery [28]. How this enzyme gets access to its substrate and what its possible role at the cell periphery may be has to be clarified.

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

Acknowledgments

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

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CHAPTER III

The impact of nonpolar lipids on the regulation of the steryl ester hydrolases Tgl1p and Yeh1p in the yeast *Saccharomyces cerevisiae*

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Published in Biochimica et Biophysica Acta (BBA) – Molecular and Cell Biology of Lipids

Volume 1862, Issue 12, Pages 1491–1501, Epub 2017 Sep 1, Print 2017 Dec

doi: 10.1016/j.bbalip.2017.08.009

Abstract

In the yeast *Saccharomyces cerevisiae* degradation of steryl esters is catalyzed by the steryl ester hydrolases Tgl1p, Yeh1p and Yeh2p. The two steryl ester hydrolases Tgl1p and Yeh1p localize to lipid droplets, a cell compartment storing steryl esters and triacylglycerols. In the present study we investigated regulatory aspects of these two hydrolytic enzymes, namely the gene expression level, protein amount, stability and enzyme activity of Tgl1p and Yeh1p in strains lacking both or only one of the two major nonpolar lipids, steryl esters and triacylglycerols. In a strain lacking both nonpolar lipids and consequently lipid droplets, Tgl1p as well as Yeh1p were present at low amount, became highly unstable compared to wild-type cells, and lost their enzymatic activity. Under these conditions both steryl ester hydrolases were retained in the endoplasmic reticulum. The lack of steryl esters alone was not sufficient to cause an altered intracellular localization of Tgl1p and Yeh1p. Surprisingly, the stability of Tgl1p and Yeh1p was markedly reduced in a strain lacking triacylglycerols, but their capacity to mobilize steryl esters remained unaffected. We also tested a possible cross-regulation of Tgl1p and Yeh1p by analyzing the behavior of each hydrolase in the absence of its counterpart steryl ester hydrolases. In summary, this study demonstrates a strong regulation of the two lipid droplet associated steryl ester hydrolases Tgl1p and Yeh1p due to the presence/absence of their host organelle.

Highlights

- The two steryl ester hydrolases Tgl1p and Yeh1p are components of lipid droplets.
- In a strain lacking lipid droplets Tgl1p-V5 and Yeh1p-V5 are retained in the ER.
- The lack of the substrate of Tgl1p and Yeh1p has no impact on their subcellular localization.
- Tgl1p-V5 and Yeh1p-V5 are highly unstable in a strain lacking lipid droplets.

Key words: yeast, lipids, steryl ester(s), sterols, hydrolase, lipid droplets

Abbreviations: ER, endoplasmic reticulum; LD, lipid droplet; QM, quadruple mutant; SE, steryl ester; TG, triacylglycerol.

1. Introduction

Sterols and fatty acids are essential lipids in all eukaryotic cells. These lipids are crucial to maintain a certain membrane permeability and fluidity. They also serve as energy source or molecules in cell signaling. An excess of sterols and free fatty acids can lead to lipotoxicity and may become harmful to the cell. One possibility to avoid such a toxic behavior, sterols and fatty acids can be stored in their biologically inert form as steryl esters (SE) and triacylglycerols (TG). Thus, SE and TG, which are mainly synthesized in the endoplasmic reticulum (ER), are stored in defined organelles named lipid droplets (LD) [1,2]. The metabolic network of synthesis and mobilization of SE and TG is important to maintain lipid homeostasis in every living cell.

In the model organism *Saccharomyces cerevisiae*, which we used for the present study, two acyl-CoA:sterol acyltransferases named Are1p and Are2p (ACAT related enzymes) are responsible for the formation of SE [3,4]. Both enzymes are located at the ER and share 49% sequence identity. Are2p is the main SE synthase under aerobic conditions with ergosterol as the preferred substrate. In cells deprived of oxygen, Are1p becomes the more important SE synthase which uses mainly ergosterol precursors as substrate [5,6]. A mutant deleted of both *ARE1* and *ARE2* completely lacks SE. Surprisingly, the depletion of SE does not impair cell growth under normal conditions.

In addition to SE, LD of the yeast *S. cerevisiae* harbor approximately the same amount of TG [7–9]. The two enzymes Dga1p, a diacylglycerol:acyl-CoA acyltransferase and Lro1p, a phospholipid:diacylglycerol acyltransferase are the key players in the formation of TG [10–13]. Dga1p is dually located to the ER and LD and contributes mostly to TG formation in the late exponential and stationary growth phase. Diacylglycerols and acyl-CoA are the substrates for Dga1p. The second major TG synthase in yeast, Lro1p, is strictly localized to the ER. This synthase is the major contributor to TG formation during exponential growth and functions in an acyl-CoA independent manner. Are1p and Are2p also contribute to the formation of TG, although to an almost negligible extent [14,15]. A yeast strain deficient of all four enzymes of SE and TG formation is devoid of LD [15]. Interestingly, the quadruple mutant *dga1*Δ*lro1*Δ*are1*Δ*are2*Δ (QM) shows no growth defect when cultivated on rich medium. Only when exogenous fatty acids are supplied a growth defect of the quadruple mutant becomes evident [16–18].

Both nonpolar lipids, TG and SE, accumulate during the stationary growth phase and are stored in LD. These LD are composed of a hydrophobic core of TG surrounded by several

shells of SE [8]. This whole particle is enclosed by a phospholipid monolayer with several associated or embedded proteins [7-9]. Upon requirement, TG and SE can be mobilized by a specific set of proteins termed TG lipases and SE hydrolases, respectively. Tgl3p, Tgl4p and Tgl5p are the three major TG lipases in *S. cerevisiae* [19–21].

Three SE hydrolases are known to mobilize SE, namely Tgl1p, Yeh1p and Yeh2p [22,23]. Tgl1p and Yeh1p were identified as paralogues of mammalian acid lipases and localize to LD. Both enzymes exhibit low *in vitro* SE hydrolase activity. The highest SE hydrolase activity was measured in the plasma membrane and attributed to Yeh2p [22]. Köffel and Schneiter [24] demonstrated that Yeh1p has a higher enzymatic activity under anaerobiosis mimicked by heme deficiency in respective mutants than under aerobic conditions. More recently, Yeh1p was also shown to be essential for starvation-induced autophagy [25]. Tgl1p, Yeh1p, and Yeh2p exhibit different substrate preferences. All three enzymes hydrolyze ergosteryl esters, but Tgl1p and Yeh2p show a slight preference for zymosteryl esters, whereas Yeh1p efficiently hydrolyses fecosteryl esters [26]. Additionally, Tgl1p was shown to contribute to TG lipolysis *in vitro*, although TG mobilization by this enzyme was low *in vivo* [27].

Studies from our laboratory revealed a regulatory effect of the presence or absence of nonpolar lipids on the behavior of TG lipases [28,29]. These findings tempted us to extend our research to two further LD resident proteins which are involved in nonpolar lipid catabolism. The present study is focused on the regulation of the two LD associated SE hydrolases Tgl1p and Yeh1p by presence or absence of one or both nonpolar lipids. For this purpose, we analyzed gene expression, protein levels as well as stability, subcellular localization and enzyme activity of Tgl1p and Yeh1p in wild type and in strains lacking SE and/or TG. Moreover, we studied regulatory effects on Tgl1p and Yeh1p by presence or absence of the counterpart SE hydrolases. In summary, our studies demonstrate that lack of TG seems to have a higher impact on Tgl1p and Yeh1p than the absence of SE.

2. Materials and Methods

2.1. Yeast strains and culture conditions

Yeast strains used in this study are listed in Table 1. Yeast cells were either grown in YPD medium containing 1% yeast extract (Oxoid Ltd, Basingstoke, UK), 2% glucose (Roth, Karlsruhe, Germany) and 2% peptone (Oxoid); or in synthetic minimal medium (SD) containing 0.67% yeast nitrogen base (ForMedium, Hundstanton, UK), 2% glucose and the respective amino acid supplements. Cells were cultivated in liquid medium at 30°C under vigorous shaking either to the exponential growth phase or to the stationary phase. Gal1 promoter-controlled genes were induced by growing cells in SD medium containing 2% galactose (ForMedium) as a carbon source. Growth was monitored by measuring the optical density at 600 nm (A_{600}).

Table 1 Yeast strains used throughout this study.

Strain	Genotype	Source
Wild type	BY4741 Mat **a**; *his3Δ1; leu2Δ0; met15Δ0; ura3Δ0*	Euroscarf
Tgl1-V5	BY4741; *TGL1-V5::HIS3MX6*	This study
Yeh1-V5	BY4741; *YEH1-V5::HIS3MX6*	This study
GFP-Tgl1	BY4741; *HIS3MX6::PGAL1-GFP* (S65T)-*TGL1*	This study
GFP-Yeh1	BY4741; *HIS3MX6::PGAL1-GFP* (S65T)-*YEH1*	This study
are1Δare2Δ	BY4742; *are1Δ::kanMX4; are2Δ::URA3*	Kindly provided by M. Spanova
are1Δare2Δ Tgl1-V5	*are1Δare2Δ; TGL1-V5::HIS3MX6*	This study
are1Δare2Δ Yeh1-V5	*are1Δare2Δ; YEH1-V5::HIS3MX6*	This study
are1Δare2Δ GFP-Tgl1	*are1Δare2Δ; HIS3MX6::PGAL1-GFP* (S65T)-*TGL1*	This study
are1Δare2Δ GFP-Yeh1	*are1Δare2Δ; HIS3MX6::PGAL1-GFP* (S65T)-*YEH1*	This study
dga1Δlro1Δ	BY4741; *dga1Δ::kanMX4; lro1Δ::kanMX4*	This study
dga1Δlro1Δ Tgl1-V5	*dga1Δlro1Δ*; *TGL1-V5::HIS3MX6*	This study
dga1Δlro1Δ Yeh1-V5	*dga1Δlro1Δ*; *YEH1-V5::HIS3MX6*	This study
dga1Δlro1Δ GFP-Tgl1	*dga1Δlro1Δ*; *HIS3MX6::PGAL1-GFP* (S65T)-*TGL1*	This study
dga1Δlro1Δ pYES2-Yeh1-GFP	*dga1Δlro1Δ*; pYES2-Yeh1-GFP	This study
QM	BY4741; *dga1Δ::kanMX4; lro1Δ::kanMX4; are1Δ::kanMX4; are2Δ::kanMX4*	Athenstaedt [30]

QM Tgl1-V5	QM; *TGL1-V5::HIS3MX6*	This study
QM Yeh1-V5	QM; *YEH1-V5::HIS3MX6*	This study
QM GFP-Tgl1	QM; *HIS3MX6::PGAL1-GFP* (S65T)-*TGL1*	This study
QM GFP-Yeh1	QM; *HIS3MX6::PGAL1-GFP* (S65T)-*YEH1*	This study
*tgl1*Δ*yeh2*Δ	BY4741; *tgl1*Δ*::kanMX4; yeh2*Δ*::kanMX4*	Wagner *et al.* [26]
*tgl1*Δ*yeh2*Δ Yeh1-V5	*tgl1*Δ*yeh2*Δ*; YEH1-V5::HIS3MX6*	This study
*tgl1*Δ*yeh2*Δ GFP-Yeh1	*tgl1*Δ*yeh2*Δ*; HIS3MX6::PGAL1-GFP* (S65T)-*YEH1*	This study
*yeh1*Δ*yeh2*Δ	BY4741; *yeh1*Δ*::kanMX4; yeh2*Δ*::kanMX4*	Wagner *et al.* [26]
*yeh1*Δ*yeh2*Δ Tgl1-V5	*yeh1*Δ*yeh2*Δ*; TGL1-V5::HIS3MX6*	This study
*yeh1*Δ*yeh2*Δ GFP-Tgl1	*yeh1*Δ*yeh2*Δ*; HIS3MX6::PGAL1-GFP* (S65T)-*TGL1*	This study
*dga1*Δ*lro1*Δ*tgl1*Δ*yeh2*Δ	BY4741; *dga1*Δ*::kanMX4; lro1*Δ*::kanMX4; tgl1*Δ*::HIS3MX6; yeh2*Δ*::URA3*	This study
*dga1*Δ*lro1*Δ*yeh1*Δ*yeh2*Δ	BY4741; *dga1*Δ*::kanMX4; lro1*Δ*::kanMX4; yeh1*Δ*::HIS3MX6; yeh2*Δ*::URA3*	This study
TM	BY4741; *tgl1*Δ*::kanMX4; yeh1*Δ*::kanMX4; yeh2*Δ*::kanMX4*	Wagner *et al.* [26]

2.2. Genetic techniques

Chromosomal tagging of *TGL1* and *YEH1* was performed by homologous recombination using the PCR-mediated method of Longtine *et al.* [31]. In brief, the inserts for the construction of Tgl1-V5, Yeh1-V5, pGAL1-GFP-Tgl1, or pGAL1-GFP-Yeh1 strains were obtained by PCR from the plasmid pFA6a-HIS3MX6-PGAL1-GFP(S65T) [31] and genomic DNA from a yeast strain containing a 6xGLY-V5-HIS3MX6 construct. Primers used for amplification of the respective DNA-fragments are listed in Table 2. Furthermore, 500 ng DNA were used for transformation of yeast strains employing the high-efficiency lithium acetate transformation protocol [32]. After transformation, cells were plated on minimal medium lacking histidine for selection and incubated for 2 to 3 days at 30°C. Positive transformants were verified by colony PCR of whole yeast cell extracts with primers listed in Table 2.

The *dga1*Δ*lro1*Δ double knock out strain was obtained by mating of the respective single deletion strains (EUROSCARF STRAIN COLLECTION), and subsequent tetrad dissection. The desired spore was verified by colony PCR of whole yeast cell extract. The

chromosomal knock out of *YEH2* and either *TGL1* or *YEH1* in the *dga1*Δ*lro1*Δ mutant background was performed by homologous recombination using the PCR-mediated method of Longtine *et al.* [31]. The knock out cassettes were obtained by PCR from the plasmids pFA6a-URA3KL or pFA6a-HIS3MX6 [28,31] with primers listed in Table 2.

The plasmid pYES2-Yeh1-GFP was constructed by homologous recombination in yeast. The pYES2 (Invitrogen, Carlsbad, CA) vector was cleaved in the multiple cloning site (MCS) by *BamH*I. The open reading frame of Yeh1-GFP was amplified by PCR using genomic DNA of a BY4741 Yeh1-GFP strain as template. The Yeh1-GFP insert and the cleaved pYES2 vector were simultaneously transformed into yeast using the lithium acetate method [32]. After transformation, cells were plated on minimal medium lacking uracil for selection and incubated for 2 to 3 days at 30°C. Positive transformants were verified by colony PCR of whole yeast cell extracts.

Table 2 Primers used throughout this study.

Primer	Sequence (5' → 3')
Fwd_Tgl1_V5tag_Cterm	AACTAGATGCCAACTCTTCGACAACTGCGCTGGATGCTCTAA ATAAAGAAAAGGGCGAGCTTCGAGGTCA
Rev_Tgl1_Ctag	TTATTATCCTAGACAAAAAATAGTTTAATAGGGTTTCTCTCGC ATTCTTTGAATTCGAGCTCGTTTAAAC
Fwd_Tgl1_Ntag	AATTCGAACAAAACTTTATTATTCTAGCACTATTTTAAAAAA CTGTCTTTTGGCAAAGAATTCGAGCTCGTTTAAAC
Rev_Tgl1_Ntag	GTATACCAAGACGACTATAATGTAATCTGTTATCGATAATCT GCCTAAAAAGGGGAAGTATTTGTATAGTTCATCCATGC
Fwd_Yeh1_V5tag_Cterm	GTTCGGACACAGAGGTGGAAACGGAGCTGGAAATGGTTGCT GAGAAGGCTAAGGGCGAGCTTCGAGGTCA
Rev_Yeh1_Ctag	TATGTATTCCCAAGTATAATTTATATTAACCTATATATCATGC TTCCTCTGAATTCGAGCTCGTTTAAAC
Fwd_Yeh1_Ntag	GGCTGCAGATAAAGTAATAGTTTTATATATAGGTATATTTAC TGCACAATTCACACGGAATTCGAGCTCGTTTAAAC
Rev_Yeh1_Ntag	ACAGCAGACTATGAACGTTGCTAGTAAATTCCTAGCTCTTTT CAACACCGCAGAAACACCTTTGTATAGTTCATCCATGC
Tgl1contr_200vS_fwd	GCTGACAATTCAAGGTTC
Yeh1contr_200vS_fwd	ATGTCAACTACAAGATCT
HIScontr_rev	TACGGGCGACAGTCACATC
Contr_Tgl1_rev	TGCACACATTTC

Contr_Yeh1_rev	GCATTGGTTCTA
Contr_N-term_GFP_fwd	CATTCTTGGACA
Yeh1-pYES2_hom_fwd	CAGCTGTAATACGACTCACTATAGGGAATATTAAGCTTGGTACCGAGCTCATGGGTGTTTCTGCGGTGTT
Yeh1-pYES2-hom-rev	TGCTGGATATCTGCAGAATTCCAGCACACTGGCGGCCGTTACTAGTCTATTTGTATAGTTCATCCA
F1_Tgl1_KO_his_Lt3	CTTTATTATTCTAGCACTATTTTAAAAAACTGTCTTTTGGCAAACGGATCCCCGGGTTAATTAA
R1_Tgl1_KO_his_Lt3	CTAGACAAAAAATAGTTTAATAGGGTTTCTCTCGCATTCTTTGAATTCGAGCTCGTTTAAA
F1_Yeh1_KO_his_Lt3	GTTTTATATATAGGTATATTTACTGCACAATTCACACGCGGATCCCCGGGTTAATTAA
R1_Yeh1_KO_his_Lt3	CAAGTATAATTTATATTAACCTATATATCATGCTTCCTCTGAATTCGAGCTCGTTTAAA
S1_Yeh2del_ura	CAACTATCATTGGGATCCCCGTGGCACCTTTACGATAAGCCGTACGCTGCAGGTCGAC
S2_Yeh2del_ura	CATATTATATTTTACAAAGAAACCACAAAGAAAAAACTTTTACCATCGATGAATTCGAGCTCG
Tgl1 fw_ctrl	CATAGGCGGTCATTTTTCTTTT
Yeh1 fw_ctrl	GAGAAGTTCTGGGGCGGTC
Yeh2 fw_ctrl	CGGTAGATGAGTTTGGAATTCAT
URA_rev_KP	GCAACCGGACCTGCATGAG

2.3. RNA isolation and real-time PCR

Total RNA isolation from cells grown to the mid logarithmic phase was performed as described by the manufacturer using the RNeasy kit from Qiagen (Hilden, Germany). After DNaseI digestion, quantitative real-time PCR was performed using SuperScript III Platinum SYBR Green One-Step qRT-PCR Kit (Invitrogen) according to the manufacturer's instructions. Reactions were performed in sealed MicroAmp Optical 96-Well Reaction Plates, and amplification was measured using an ABI 7500 instrument (Applied Biosystems). Samples were quantified using the $\Delta\Delta C_t$ method described by Livak and Schmittgen [33]. *ACT1* served as internal control. Primers used for real-time PCR are listed in Table 3.

Table 3 Primers used for qRT-PCR.

Primer	Sequence (5' → 3')
TGL1_RTFw	GACATGGAAGACAACTCATCCAACGCATG
TGL1_RTRev	GACCTCATCCGCTTCATCAGCTTGTAC
YEH1_RTFw	GAAGTTGATGCAATGGTGGCTGTCAC
YEH1_RTRev	GTCTGAAAACCACTTAACATTGTCGGGGAAC
ACT1_RTFw	CCAGCCTTCTACGTTTCCATCCAAG
ACT1_RTRev	GACGTGAGTAACACCATCACCGGA

2.4. Isolation and characterization of microsomes and lipid droplets

With the exceptions of QM Yeh1-V5 and QM Tgl1-V5 which were grown to the mid logarithmic phase all other strains were grown to the stationary phase. Isolation of highly pure microsomes and LD was performed as described previously [7,34]. The protein concentration was analyzed by the method of Lowry *et al.* [35] using bovine serum albumin as a standard. Prior to protein quantification, samples of LD fractions were delipidated according to the method of Wessel and Flügge [36]. Afterwards, proteins were precipitated with trichloroacetic acid at a final concentration of 10% and solubilized in 0.1% SDS, 0.1% NaOH. Proteins were separated by SDS-PAGE using 10% polyacrylamide gels according to the method of Laemmli [37]. Western blot analysis was performed as described by Haid and Suissa [38]. 10 µg of proteins from each fraction were loaded, and proteins were detected using mouse or rabbit antisera as primary antibody. The antibodies used were directed against the V5-tag (Invitrogen); glyceraldehyde-3-phosphate dehydrogenase (GAPDH; cytosolic marker); Wbp1p or 40 kDa protein (ER-marker), and Erg6p or Ayr1p (LD-marker). Primary antibodies were detected with peroxidase-conjugated goat anti-mouse or anti-rabbit IgG, respectively, as second antibody and SuperSignal® West Pico Chemiluminescent substrate solution (Thermo Scientific, Rockford, Illinois, USA) or Amersham™ ECL Select™ Western blotting Detection Reagent (GE Healthcare, Buckinghamshire, UK).

2.5. Determination of protein stability

To test protein stability, protein synthesis was blocked by adding cycloheximide at a final concentration of 100 µg/ml to cells grown to the mid logarithmic growth phase in YPD at 30°C. At time points indicated, aliquots of cells were harvested by centrifugation at 17,000

x g for 10 min at 4°C. To disintegrate the cells, the cell pellet was suspended in lysis buffer (1.85 M NaOH, 7.5% ß-mercaptoethanol) and incubated for 10 min on ice. After precipitating the proteins with trichloroacetic acid at a final concentration of 10%, the samples were centrifuged at 17,000 x g at 4°C for 10 min, the pellet was washed twice with ice-cold distilled water and dissolved in Laemmli buffer [37] by incubation at 37°C for 30 min. Gel electrophoresis and Western blot analysis were performed as described above (see Section 2.4). Quantification of the relative intensities of the immunoreactive bands was accomplished using ImageJ software.

2.6. Fluorescence microscopy

Yeast strains were grown at 30°C in SD medium (see Section 2.1) to the late logarithmic growth phase. To induce the expression of the recombinant proteins, an aliquot of the culture was shifted to galactose containing SD medium for 6-8 h. In case of the *dga1*Δ*lro1*Δ strain containing the pYES2-Yeh1-GFP plasmid, cells were grown in SD medium containing 2% raffinose instead of glucose to ensure rapid protein expression after the shift to galactose medium. The localization of Yeh1p-GFP in the *dga1*Δ*lro1*Δ strain was monitored after 4-5 h. Fluorescence microscopy was carried out on a Zeiss Axioskop microscope using a ×100 oil immersion objective with a narrow band enhanced GFP (eGFP) filter (Zeiss). Images were taken with a Visicam CCD camera and displayed using the Metamorph Imaging software (Visitron Systems, Puchheim, Germany). The exposure time for visualization was 5000 ms for ER proteins and 300 ms for LD proteins. In case of the *dga1*Δ*lro1*Δ pYES2-Yeh1-GFP strain, the exposure time to visualize LD protein was 8000 ms. Transmission images were obtained by using Nomarski optics (differential interference contrast).

2.7. Enzyme analysis

The SE hydrolase activity *in vitro* was measured as described previously [22,39,40]. In brief, for substrate preparation, 16.5 µl of cholesteryl oleate (1 mg/ml in chloroform/methanol; 2:1, v/v), 2 µl of [1-alpha, 2-alpha (n)-^{3}H]cholesteryl oleate (43.0 Ci/mmol, 1.59 TBq/mmol; Amershan BioSciences UK Ltd. Buckinghamshire, UK) and 15 µl of Triton X-100 (100 mg/ml in acetone) were mixed and taken to complete dryness under a stream of nitrogen. The remnant was suspended in 250 µl 0.1 M Tris/Cl^{-} buffer (pH 7.4), and

the reaction was initiated by the addition of 250 µl of either microsomal fractions (250-400 µg protein) or highly purified LD (15-30 µg protein) as an enzyme source. After incubation for 2 min at 30°C, the reaction was stopped by adding 3 ml of chloroform/methanol (2:1, v/v). Then, lipids were extracted according to Folch *et al.* [41] and separated by thin-layer chromatography (TLC) using the solvent system light petroleum/diethyl ether/acetic acid (35:15:1, per vol.). Bands corresponding to cholesterol were scraped off the plate, and radioactivity was measured by liquid szintillation counting using LSC Safety (Baker, Deventer, Netherlands) with 5% water as scintillation cocktail.

The *in vivo* mobilization of SE was determined according to Leber *et al.* [42] and Wagner *et al.* [26]. In brief, yeast cells were grown in synthetic complete medium to the stationary phase, and terbinafine, an inhibitor of fungal squalene epoxidase, dissolved in ethanol was added at a final concentration of 30 µg/ml. At time points indicated, aliquots of the culture were withdrawn, and lipids of total cells extracted by the method of Folch *et al.* [41]. After separation by TLC using the solvent system light petroleum/diethyl ether/acetic acid (35:15:1, per vol.) lipids were visualized by dipping the TLC plate in a solution consisting of 0.63 g of $MnCl_2$.4 H_20, 60 ml water, 60 ml methanol and 4 ml concentrated sulfuric acid, and heating at 105°C for 30 min. Bands according to SE were quantified by densitometric scanning with a CAMAG TLC Scanner 3 at 600 nm and comparison with authentic standards containing defined amounts of the respective lipid.

3. Results

3.1. Protein levels of Tgl1p and Yeh1p in cells lacking either one or both nonpolar lipid species

To investigate the fate of the two LD associated SE hydrolases Tgl1p and Yeh1p in the absence of SE and/or TG we determined gene expression and protein levels of genomically V5-tagged versions in the respective strain backgrounds. The *are1*Δ*are2*Δ mutant lacks SE, and *dga1*Δ*lro1*Δ is unable to synthesize TG, but both strains still contain LD. The quadruple mutant *dga1*Δ*lro1*Δ*are1*Δ*are2*Δ (QM) is devoid of LD, because this strain is unable to produce both SE and TG. First, we analyzed gene expression of *TGL1* and *YEH1* in these strain backgrounds (Figure 1 A). In strains lacking either SE or TG, the mRNA level of *TGL1* was only marginally reduced. However, in the QM gene expression of *TGL1* was

reduced to approximately 50% of wild-type level (Figure 1 A, left). Expression of *YEH1* was reduced to 70% of wild-type level in a strain lacking SE (*are1*Δ*are2*Δ), whereas in *dga1*Δ*lro1*Δ and QM the *YEH1* mRNA level drop to about 50% of the wild type (Figure 1 A, right).

Next, we investigated the protein amount of Tgl1p and Yeh1p in the different strain backgrounds. As shown in Figure 1 B the reduced mRNA level of *TGL1* and *YEH1* in the QM compared to wild-type control was paralleled by a strongly reduced amount of the respective proteins. We observed also a slightly reduced protein level of Tgl1p in the strain which is unable to synthesize TG (*dga1*Δ*lro1*Δ). Surprisingly, the amount of Yeh1p seemed to be unaffected in strains lacking SE (*are1*Δ*are2*Δ) or TG (*dga1*Δ*lro1*Δ), although the mRNA level was markedly reduced in the respective strains (Figure 1 A and B, right). Hence, the levels of the translation products did not completely match the levels of transcription.

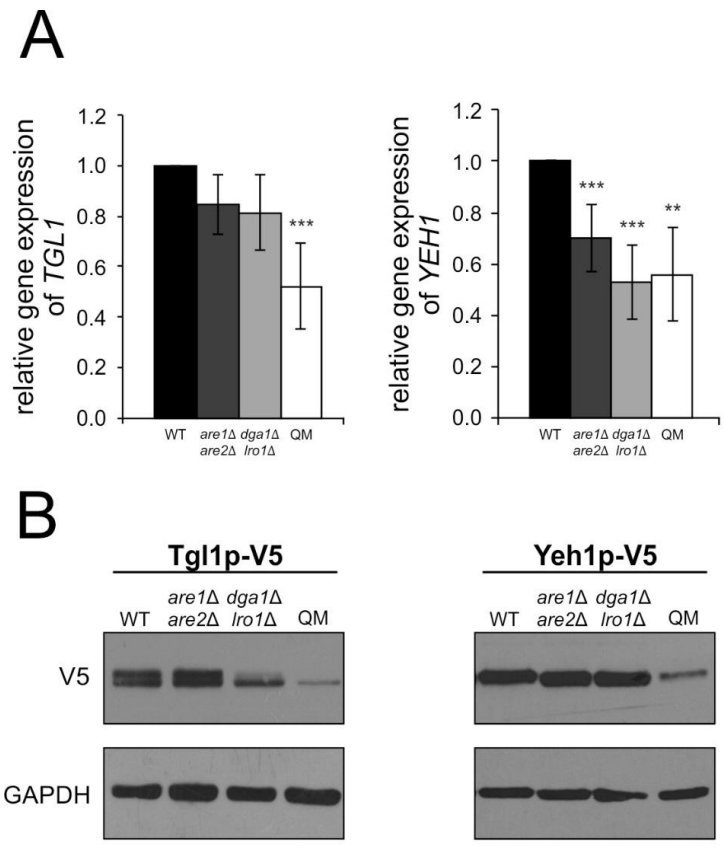


Figure 1 Gene expression and protein level of Tgl1p and Yeh1p in cells defective in nonpolar lipid synthesis.

(A) The relative gene expression of *TGL1* (left) and *YEH1* (right) in wild type (WT) (black bar), *are1*Δ*are2*Δ (dark grey bar), *dga1*Δ*lro1*Δ (grey bar) and *dga1*Δ*lro1*Δ*are1*Δ*are2*Δ (QM) (white bar) strains was quantified by real-time PCR. Wild-type values were set at 1. Data are mean values from at least three independent experiments with the respective standard

deviations as shown. Statistical significance was determined using Student's unpaired *t*-test, ***$p < 0.001$, **$p < 0.005$. **(B)** Protein analysis of V5-tagged variants of Tgl1p and Yeh1p from total cell extracts of wild type, *are1*Δ*are2*Δ, *dga1*Δ*lro1*Δ and QM strains grown to the mid logarithmic growth phase is shown. The primary antibody was directed against the V5-tag (V5). The cytosolic marker protein glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as loading control. Western blots shown are one representative of three independent experiments.

3.2. Protein stability of Tgl1p and Yeh1p in cells lacking either one or both nonpolar lipid species

As Tgl1p and Yeh1p showed different protein amounts in wild type, *are1*Δ*are2*Δ, *dga1*Δ*lro1*Δ and the QM strain, we wondered whether the two LD resident SE hydrolases revealed alterations in their protein stability. Thus, we investigated the stability of Tgl1p-V5 and Yeh1p-V5 in the presence or absence of nonpolar lipids by blocking new protein synthesis with cycloheximide and analyzing the remaining amounts of Tgl1p-V5 and Yeh1p-V5 at different time points. We observed a similar behavior of Tgl1p and Yeh1p regarding their stability in the different strain backgrounds (Figure 2 and Figure 3). Both proteins lost their stability over time to a certain degree in wild-type cells (see black bars in Figure 2 E and Figure 3 E). In the *are1*Δ*are2*Δ strain lacking SE, the stability of Tgl1p-V5 and Yeh1p-V5 was only slightly reduced compared to wild type (Figure 2 B, E and Figure 3 B, E). Interestingly, in the absence of TG (*dga1*Δ*lro1*Δ) both SE hydrolases lost significantly more of their protein over time than in the absence of SE (Figure 2 C, E and Figure 3 C, E). The QM, which lacks both nonpolar lipid species and therefore LD, showed a cumulative effect on protein stability and a very short protein half-life of Tgl1p-V5 and Yeh1p-V5 (Figure 2 D, E and Figure 3 D, E). Minor differences between the two proteins could be seen under wild-type conditions. Yeh1p-V5 was slightly less stable than Tgl1p-V5 (Figure 3 A, E and Figure 2 A, E). In summary, the stability of the two LD resident SE hydrolases was significantly compromised in strains lacking LD (QM). Moreover, an unexpected instability of Tgl1p-V5 and Yeh1p-V5 was already observed in the absence of TG only.

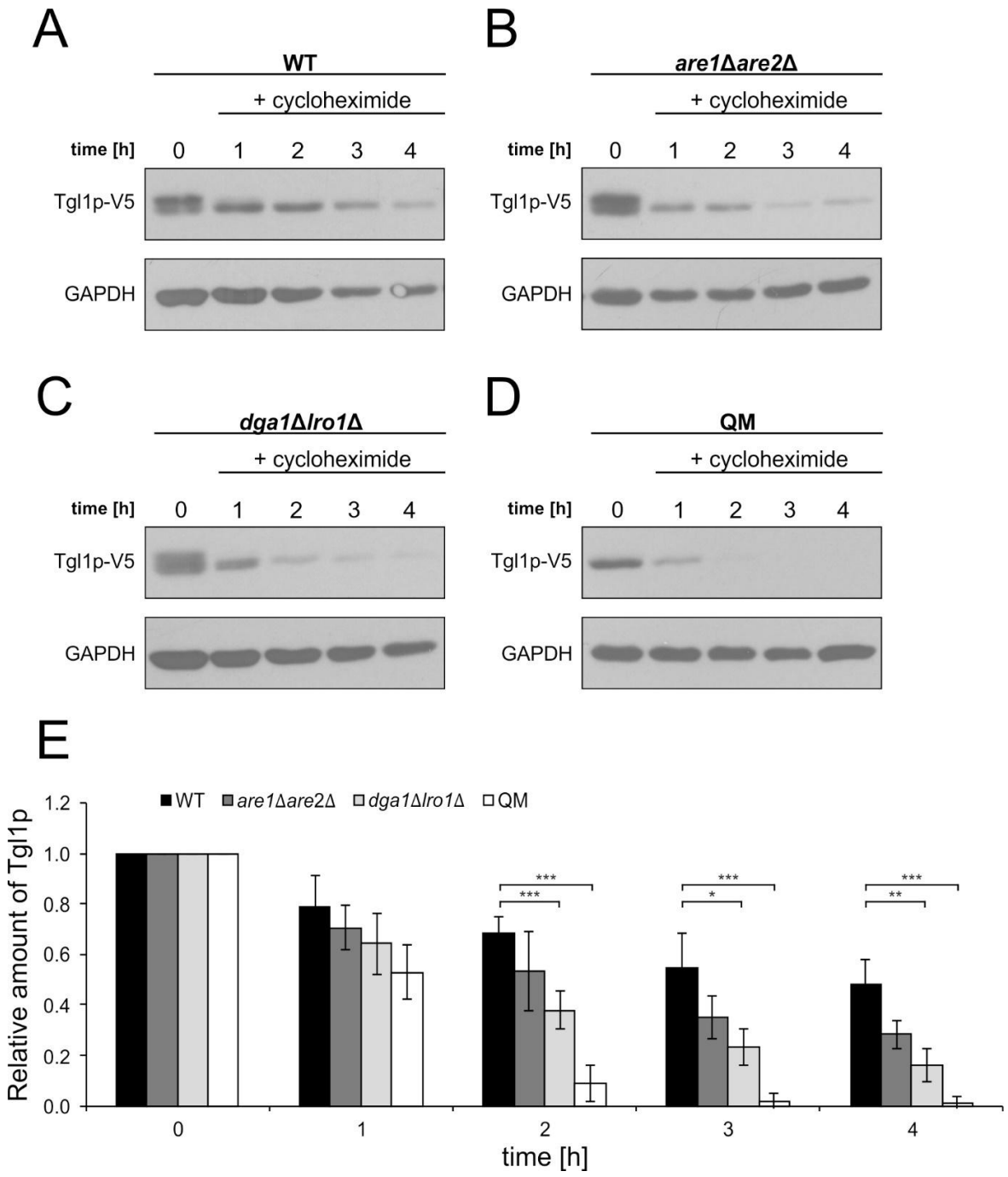


Figure 2 Protein stability of Tgl1p-V5 in cells defective in nonpolar lipid synthesis.

Western blot analysis of Tgl1p-V5 was performed with total cell extracts from **(A)** wild type (WT), **(B)** *are1*Δ*are2*Δ, **(C)** *dga1*Δ*lro1*Δ and **(D)** QM strains grown for times as indicated after addition of 100 µg/ml cycloheximide to cells grown to the mid logarithmic growth phase. The primary antibody was directed against the V5-tag (V5). The cytosolic marker protein glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as loading control. Western blots shown are one representative experiment of at least four independent experiments. **(E)** Relative amounts of V5-tagged variants of Tgl1p analyzed by Western

blotting were calculated using the ImageJ software. The amount of Tgl1p-V5 at time point 0 (addition of cycloheximide) was set at 1. Black bars represent the protein amount of Tgl1p-V5 in wild type, dark grey bars in *are1*Δ*are2*Δ, grey bars in *dga1*Δ*lro1*Δ and white bars in QM strains at the respective time points. Data are mean values from at least four independent experiments with the respective standard deviations as shown. Statistical significance was determined using Student's unpaired *t*-test, ***$p < 0.001$, **$p < 0.005$, *$p < 0.01$.

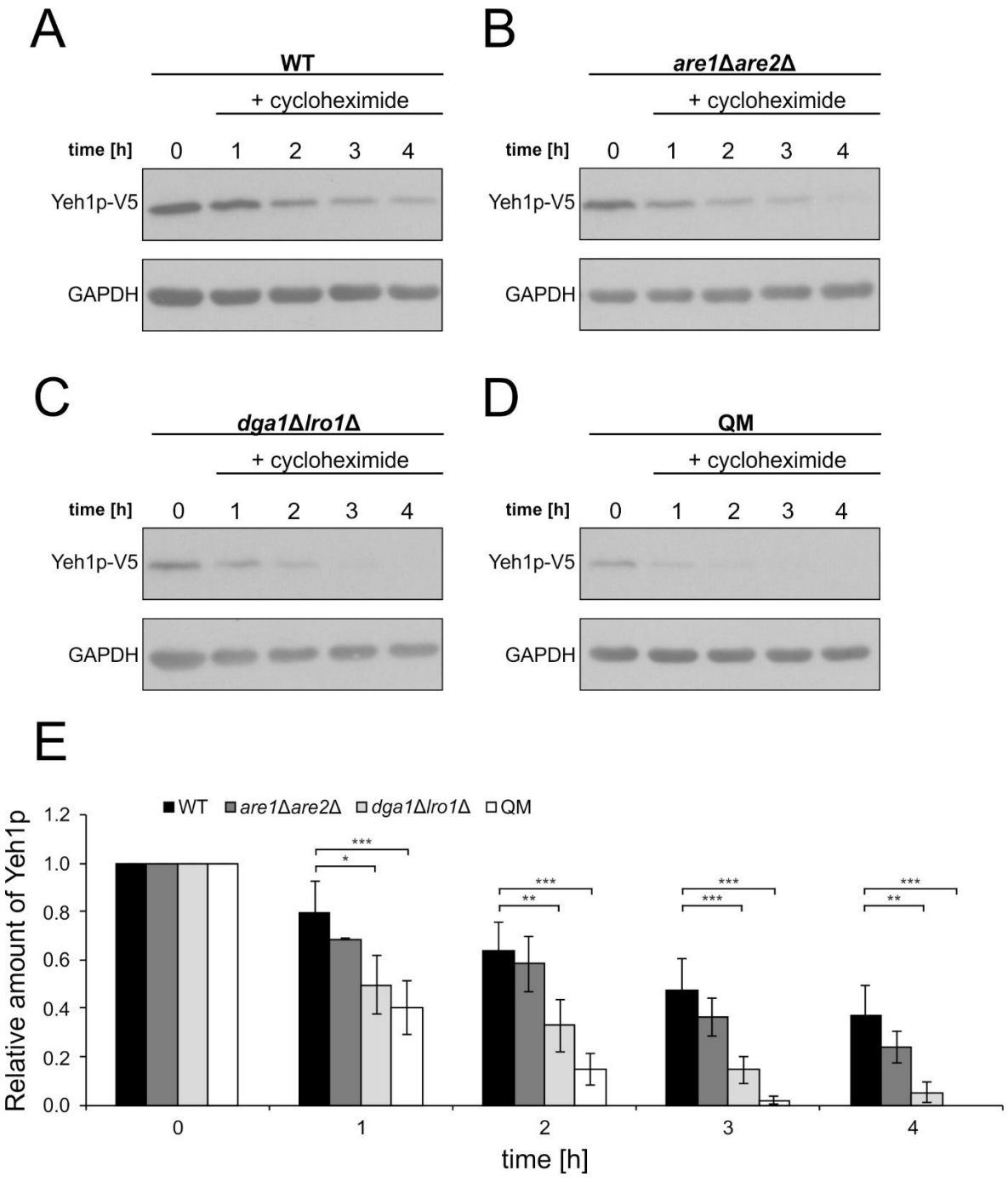


Figure 3 Protein stability of Yeh1p-V5 in cells defective in nonpolar lipid synthesis.
Western blot analysis of Yeh1p-V5 was performed with total cell extracts from **(A)** wild type (WT), **(B)** *are1*Δ*are2*Δ, **(C)** *dga1*Δ*lro1*Δ and **(D)** QM strains grown for times as indicated after addition of 100 µg/ml cycloheximide to cells grown to the mid logarithmic growth phase. The primary antibody was directed against the V5-tag (V5). The cytosolic marker protein glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as loading control. Western blots shown are one representative experiment of at least three independent experiments. **(E)** Relative amounts of V5-tagged variants of Yeh1p analyzed by Western blotting were calculated using the ImageJ software. The amount of Yeh1p-V5 at time point 0 (addition of cycloheximide) was set at 1. Black bars represent the protein amount of Yeh1p-V5 in wild type, dark grey bars in *are1*Δ*are2*Δ, grey bars in *dga1*Δ*lro1*Δ and white bars in QM strains at the respective time points. Data are mean values from at least four independent experiments with the respective standard deviations as shown. Statistical significance was determined using Student's unpaired *t*-test, ***$p < 0.001$, **$p < 0.005$, *$p < 0.01$.

3.3. In the absence of lipid droplets, Tgl1p and Yeh1p are retained in the endoplasmic reticulum

Köffel *et al.* [23] identified Tgl1p and Yeh1p as LD proteins in wild-type strains. However, several other studies demonstrated a dual localization of proteins to the ER and LD underlining a close relationship of these two organelles [12,43,44]. It is also known from previous studies that LD resident proteins relocalize to the ER in mutant strains lacking LD [28,29,45,46]. To test, whether this is also the case for the two LD resident SE hydrolases, we examined the subcellular localization of Tgl1p-V5 and Yeh1p-V5 in wild type, *are1*Δ*are2*Δ, *dga1*Δ*lro1*Δ, and the QM. Cell fractionation and Western blot analysis revealed that Tgl1p-V5 and Yeh1p-V5 were exclusively present in the LD fraction in wild-type cells (Figure 4 A and Figure 5 A). As expected, the localization of Tgl1p-V5 and Yeh1p-V5 shifted towards the total microsomal fraction in cells lacking LD (Figure 4 D and Figure 5 D). Thus, under these conditions these two proteins are retained in the ER and behave like other LD proteins [28,29]. We also analyzed the localization of the two SE hydrolases in strains lacking either SE (*are1*Δ*are2*Δ) or TG (*dga1*Δ*lro1*Δ), because recently we have shown that the subcellular localization of the TG lipase Tgl5p shifted slightly towards microsomal fractions in cells lacking its substrate (*dga1*Δ*lro1*Δ) [29]. However, the localization of Tgl1p-V5 and Yeh1p-V5 was neither affected by the absence of SE nor of TG. In the *are1*Δ*are2*Δ as well as in the

*dga1*Δ*lro1*Δ strain both SE hydrolases localized to the LD fraction exclusively (Figure 4 B, C and Figure 5 B, C).

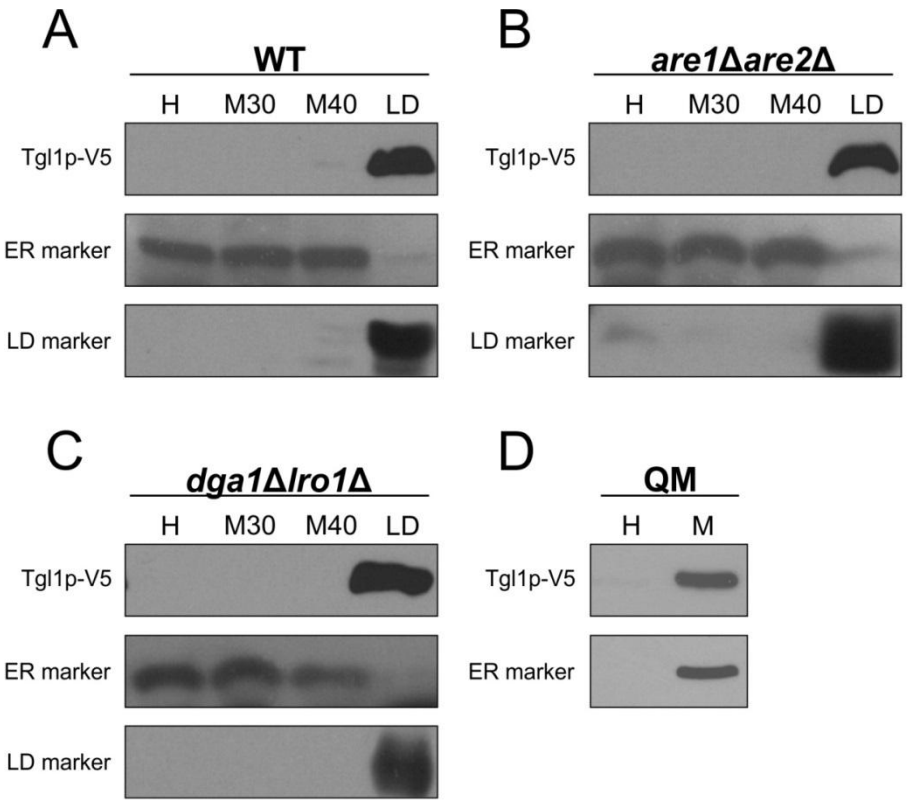


Figure 4 Localization of Tgl1p-V5 in cells defective in nonpolar lipid synthesis.

Western blot analysis of Tgl1p-V5 was performed with homogenate (H), 30,000 x g microsomal (M30), 40,000 x g microsomal (M40), lipid droplet (LD) fractions from **(A)** wild type (WT), **(B)** *are1*Δ*are2*Δ and **(C)** *dga1*Δ*lro1*Δ strains grown to the stationary growth phase. **(D)** Homogenate (H) and microsomes (M) of the QM grown to the mid logarithmic phase. Primary antibodies were directed against the V5-tag (Tgl1p-V5), Wbp1p or 40 kDa protein (ER marker), and Erg6p or Ayr1p (LD marker). Western blot analyses are representative of at least two independent experiments.

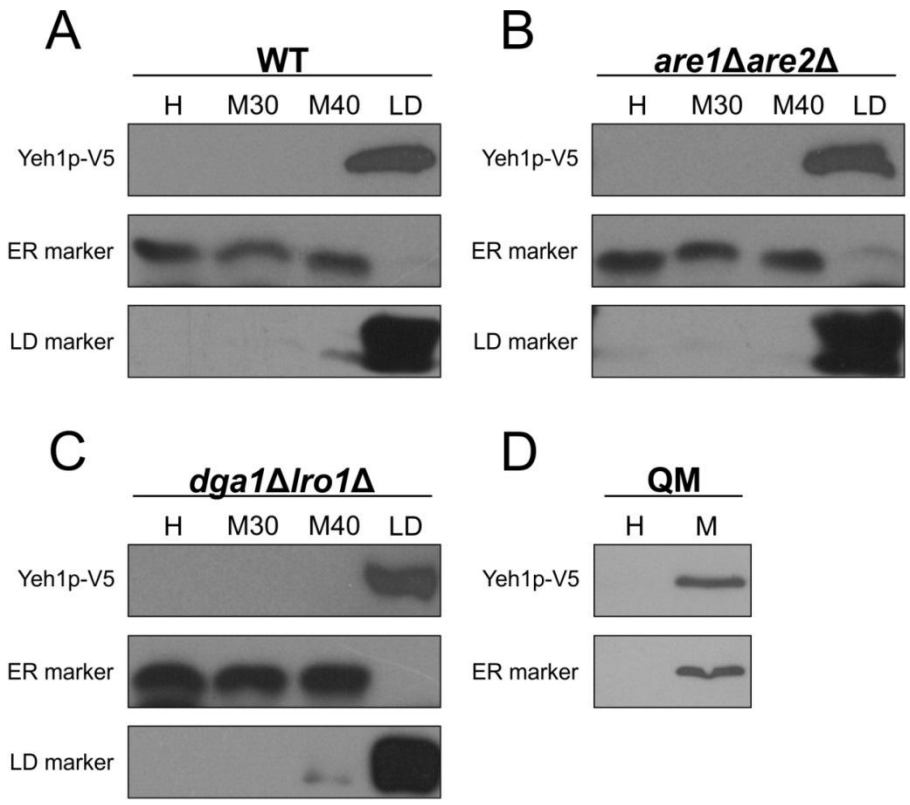


Figure 5 Localization of Yeh1p-V5 in cells defective in nonpolar lipid synthesis.
Western blot analysis of Yeh1p-V5 was performed with homogenate (H), 30,000 x g microsomal (M30), 40,000 x g microsomal (M40), lipid droplet (LD) fractions from **(A)** wild type (WT), **(B)** *are1*Δ*are2*Δ and **(C)** *dga1*Δ*lro1*Δ strains grown to the stationary growth phase. **(D)** Homogenate (H) and microsomes (M) of the QM grown to the mid logarithmic phase. Primary antibodies were directed against the V5-tag (Yeh1p-V5), Wbp1p or 40 kDa protein (ER marker), and Erg6p or Ayr1p (LD marker). Western blot analyses are representative of at least two independent experiments.

The localization of Tgl1p and Yeh1p in the different strain backgrounds was confirmed by fluorescence microscopy. In Figure 6 and Figure 7, the punctuate structures in wild type, *are1*Δ*are2*Δ and dga*1*Δ*lro1*Δ represent the fluorescence signal from the GFP-tagged Tgl1p and Yeh1p variants. In differential interference contrast (DIC) LD become visible as small bumps on the cell surface (highlighted with arrow heads). The respective pattern in DIC perfectly matches with the GFP signal, which confirms the LD localization of Tgl1p and Yeh1p in wild type, *are1*Δ*are2*Δ and dga*1*Δ*lro1*Δ. In the QM which lacks LD Tgl1p and Yeh1p were found to be enriched in the nuclear ER as shown in the bottom

pictures of Figure 6 and Figure 7. Thus, data obtained with fluorescence microscopy reflect the results of cell fractionation and Western blotting despite the use of different constructs. For fluorescence microscopy we made use of N-terminally GFP-tagged proteins under the control of the Gal1-promoter, because the signal for the C-terminally GFP-tagged proteins under their endogenous promoter was too weak to be analyzed, most likely due to the low expression levels of Tgl1p and Yeh1p. In summary, we can conclude that the absence of SE, the substrate of our proteins of interest, is not sufficient to affect their subcellular distribution. Only in strains lacking LD Tgl1p and Yeh1p relocalize to the ER.

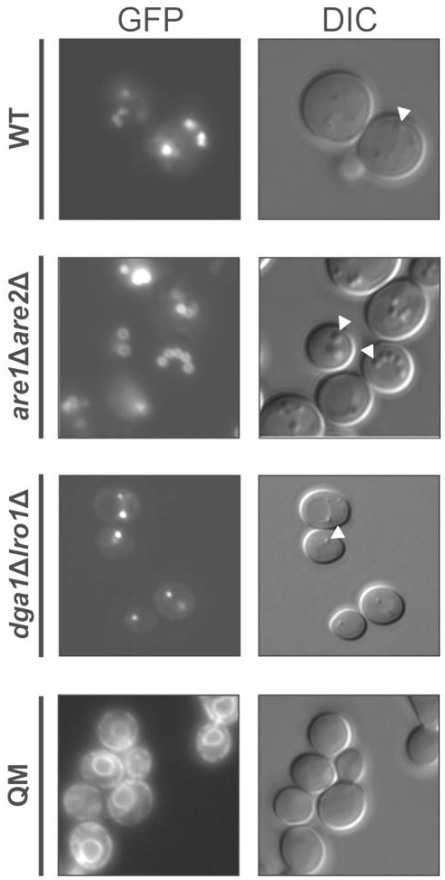


Figure 6 Fluorescence microscopy of GFP-tagged variants of Tgl1p in cells defective in nonpolar lipid synthesis.

Fluorescence microscopy of pGAL1-GFP-Tgl1 in wild type (WT), *are1*Δ*are2*Δ, *dga1*Δ*lro1*Δ and *dga1*Δ*lro1*Δ*are1*Δ*are2*Δ (QM) grown to the mid to late logarithmic growth phase after induction with galactose for 6-8 hours is shown. DIC, differential interference contrast. Arrow heads are indicating LD.

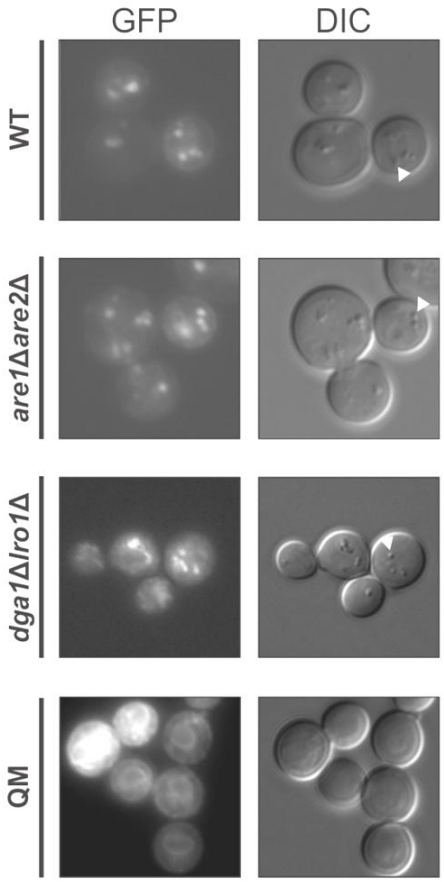


Figure 7 Fluorescence microscopy of GFP-tagged variants of Yeh1p in cells defective in nonpolar lipid synthesis.

Fluorescence microscopy of pGAL1-GFP-Yeh1 in wild type (WT), *are1*Δ*are2*Δ and *dga1*Δ*lro1*Δ*are1*Δ*are2*Δ (QM) grown to the mid to late logarithmic growth phase after induction with galactose for 6-8 hours is shown. For *dga1*Δ*lro1*Δ cells, the fluorescence signal arises from the galactose inducible construct pYES2-Yeh1-GFP. DIC, differential interference contrast. Arrow heads are indicating LD.

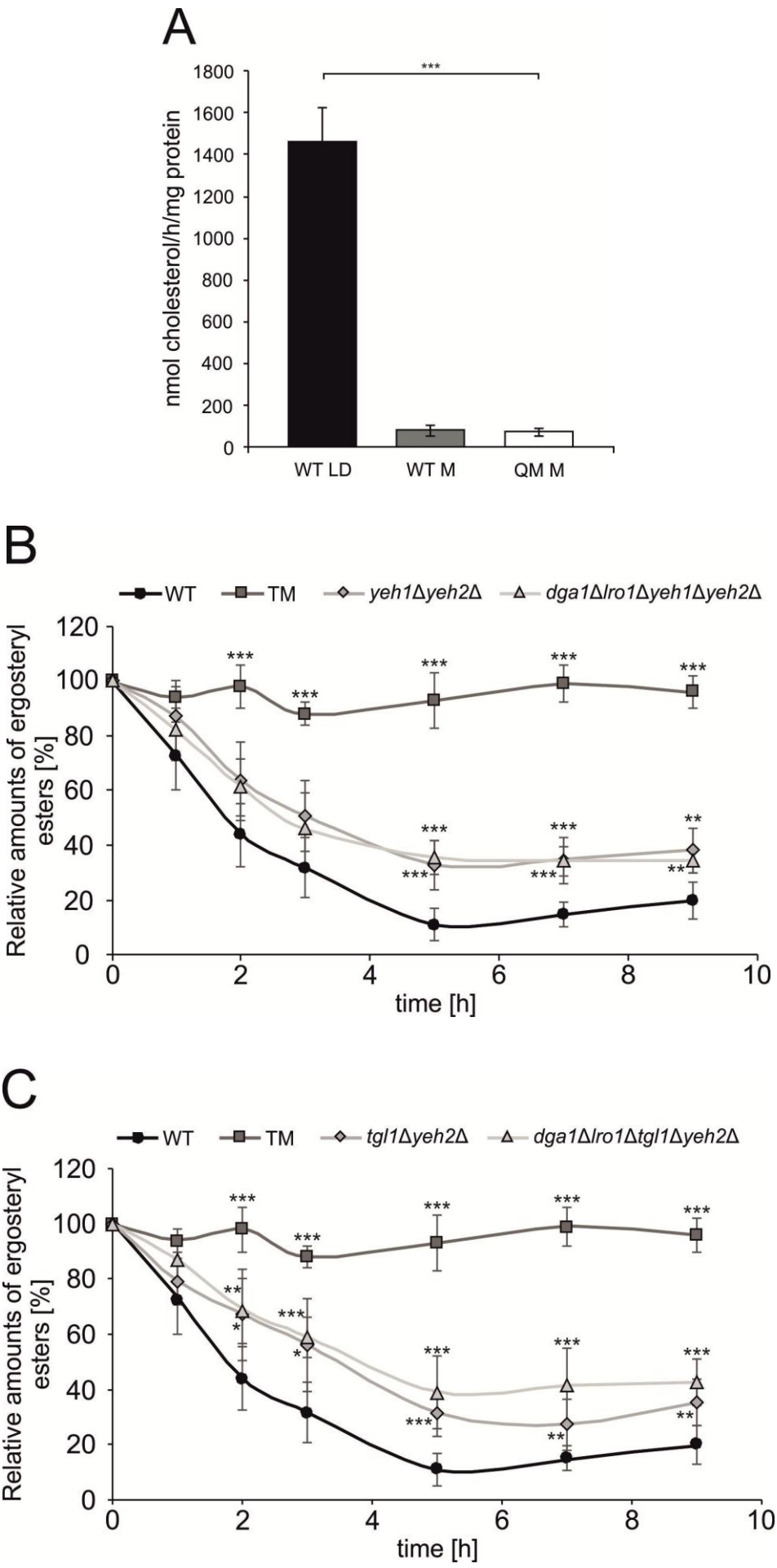
3.4. Enzyme activity of Tgl1p and Yeh1p in mutant cells lacking triacylglycerols and/or lipid droplets

The fact that Tgl1p and Yeh1p are retained in the ER from a strain lacking LD raised the question concerning the activity of the two SE hydrolases in the ER versus LD. To test for SE hydrolase activity of Tgl1p and Yeh1p retained in the ER, we performed *in vitro* assays tracing the liberation of radiolabelled cholesterol from [1-alpha, 2-alpha (n)-^{3}H] cholesteryl oleate. As a positive control we used the LD fraction from wild type which contains Tgl1p and Yeh1p as enzyme source; and as negative control we utilized the microsomal fraction

from wild type which lacks both SE hydrolases. These control activities were compared with the activity found in the microsomal fraction from the QM strain. As shown in Figure 8 A, the activity associated with the microsomal fraction of the QM, which contains Tgl1p and Yeh1p, was similar to that of wild type which lacks both SE hydrolases. These data indicate that Tgl1p and Yeh1p present in microsomes are not able to contribute to SE hydrolysis.

As Tgl1p and Yeh1p are quite unstable in a strain lacking exclusively TG similar to the QM strain lacking both TG and SE (see Figures 2 and 3), we wondered whether the absence of TG alone already affected the capacity of Tgl1p and/or Yeh1p to mobilize SE. Therefore, we performed *in vivo* assays monitoring SE mobilization in TG deficient strains after the addition of terbinafine, an inhibitor of fungal squalene epoxidase which forces the cells to use up their SE stores for ongoing membrane formation. To test the impact of TG on the hydrolytic activity of Tgl1p and Yeh1p separately, we used the strains *yeh1*Δ*yeh2*Δ and *dga1*Δ*lro1*Δ*yeh1*Δ*yeh2*Δ to test for Tgl1p, and *tgl1*Δ*yeh2*Δ and *dga1*Δ*lro1*Δ*tgl1*Δ*yeh2*Δ to test for Yeh1p. Wild type and the triple mutant *tgl1*Δ*yeh1*Δ*yeh2*Δ (TM) lacking all three SE hydrolases served as positive and negative control, respectively. As expected, both *yeh1*Δ*yeh2*Δ (Tgl1p active) and *tgl1*Δ*yeh2*Δ (Yeh1p active) mobilized SE to a lesser extent than the wild type control (Figure 8 B and C). The corresponding mutant strains lacking TG, *dga1*Δ*lro1*Δ*yeh1*Δ*yeh2*Δ and *dga1*Δ*lro1*Δ*tgl1*Δ*yeh2*Δ, also mobilized SE. As the mobilization rate of SE in the TG deficient quadruple mutants was similar to their corresponding double mutant strains, lack of TG obviously does not compromise the hydrolytic function of Tgl1p and Yeh1p *in vivo*.

In summary, our analyses revealed that the capacity of Tgl1p and Yeh1p to hydrolyze SE is lost when the proteins mislocalize to the ER, but not in the absence of TG only.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Relative amounts of ergosteryl esters [%]
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Figure 8 *In vitro* and *in vivo* enzyme activity of Tgl1p and Yeh1p.

(A) *In vitro* SE hydrolase activity of wild-type lipid droplets (WT LD; black bar), wild-type microsomes (WT M; grey bar) and QM microsomes (QM M; white bar). Data are mean values from at least three independent experiments with the respective standard deviations as shown. Statistical significance was determined using Student's unpaired *t*-test, ***$p < 0.001$. *In vivo* SE mobilization measured in **(B)** wild type (●), TM *tgl1*Δ*yeh1*Δ*yeh2*Δ (■), *yeh1*Δ*yeh2*Δ (◊), and *dga1*Δ*lro1*Δ*yeh1*Δ*yeh2*Δ (Δ); and **(C)** wild type (●), TM *tgl1*Δ*yeh1*Δ*yeh2*Δ (■), *tgl1*Δ*yeh2*Δ (◊), and *dga1*Δ*lro1*Δ*tgl1*Δ*yeh2*Δ (Δ) is shown. After the addition of terbinafine aliquots of the respective cell cultures were withdrawn at the indicated time points, and the amount of SE was determined (see Section 2.7). Data from **(B)** and **(C)** are mean values from at least four independent experiments in double estimation with the respective standard deviations as shown. Statistical significance was determined using Student's unpaired *t*-test, ***$p < 0.001$, **$p < 0{,}005$, *$p < 0{,}01$.

3.5. Regulatory interaction of the two LD localized SE hydrolases Tgl1p and Yeh1p

Previous results from our laboratory demonstrated that SE accumulate in cells lacking *TGL1*, but not in a *yeh1*Δ strain [26,47]. To examine a potential crosstalk between cellular SE hydrolases we investigated the behavior of Tgl1p and Yeh1p in *yeh1*Δ*yeh2*Δ and *tgl1*Δ*yeh2*Δ. Yeh2p is the third SE hydrolase of the yeast, but is a component of the plasma membrane [22]. We used the double deletion strains to exclude an influence of Yeh2p on our results. Unexpectedly, the mRNA level (Figure 9 A) and the protein amount (Figure 9 B) of Tgl1p-V5 was neither up- nor downregulated in a *yeh1*Δ*yeh2*Δ strain which lacks both counterpart SE hydrolases. The protein stability of Tgl1p-V5 was also unaffected by the absence of the other two SE hydrolases (Figure 9 C, D). For Yeh1p-V5, a 3-fold upregulation of the mRNA was measured in the *tgl1*Δ*yeh2*Δ strain compared to wild type (Figure 10 A). To our surprise, the amount of protein was hardly affected by the background mutation (Figure 10 B). In the *tgl1*Δ*yeh2*Δ strain, Yeh1p-V5 is as stable as in wild-type cells (Figure 10 C, D). Also the subcellular localization of Tgl1p and Yeh1p, respectively, was not affected by the absence of their counterpart SE hydrolases. Both proteins remain associated with the LD as proofed by Western blot analysis (Figure 11 A, C) and fluorescence microscopy (Figure 11 B, D). These results suggest that regarding the protein amount and protein stability as well as the subcellular localization there is no crosstalk regulation between Tgl1p and Yeh1p.

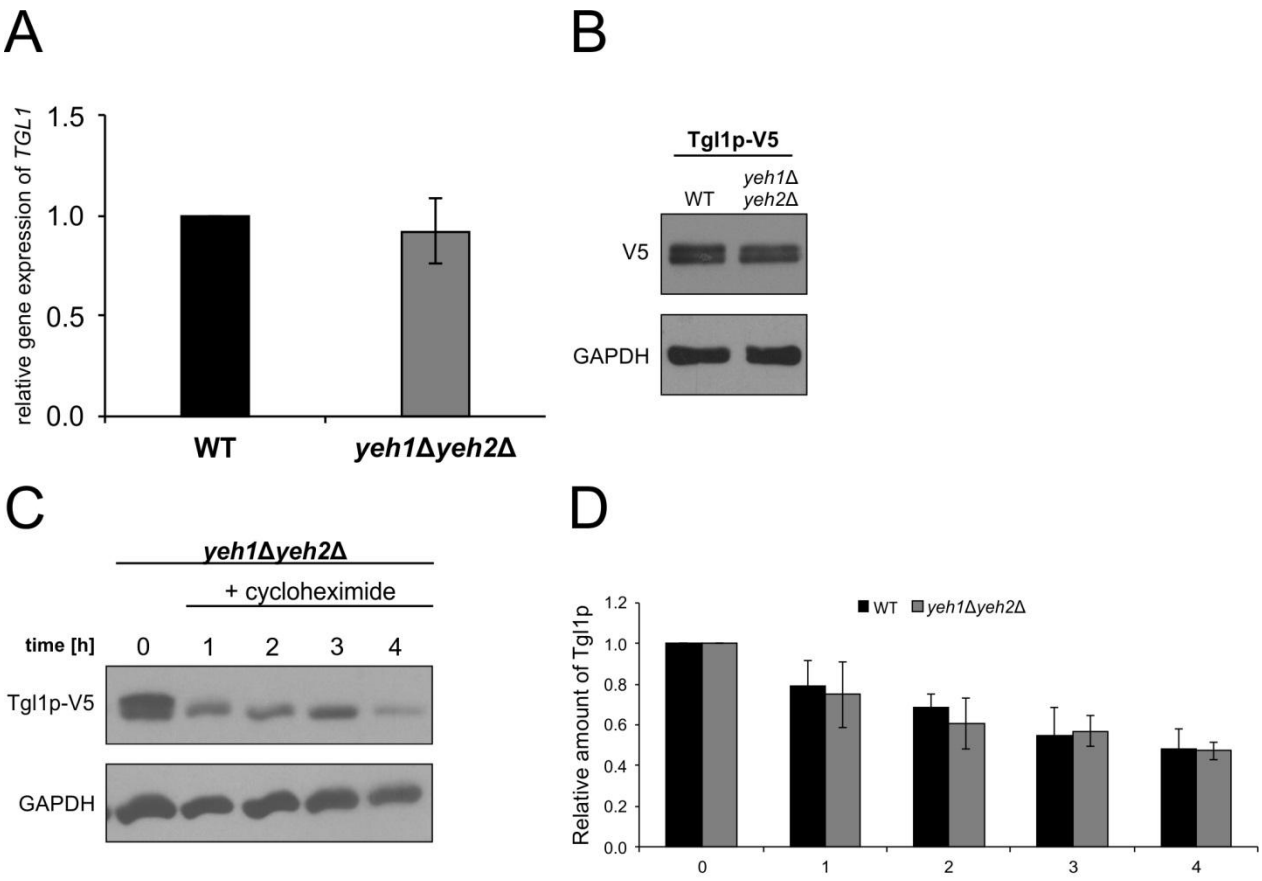

Figure 9 Gene expression, protein level and stability of Tgl1p in cells defective in both counterpart SE hydrolases.

Relative gene expression of **(A)** *TGL1* in wild type (WT) (black bar) and *yeh1*Δ*yeh2*Δ (dark grey bar) was measured by real-time PCR. The wild-type value was set at 1. Data are mean values from at least three independent experiments with the respective standard deviations as shown. **(B)** Protein analysis of V5-tagged Tgl1p from total cell extracts of wild type and *yeh1*Δ*yeh2*Δ grown to the mid logarithmic growth phase. The primary antibody was directed against the V5-tag (V5). The cytosolic marker protein glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as loading control. Western blot analysis shown is one representative of three independent experiments. To test protein stability of Tgl1p-V5 in the absence of both respective SE hydrolases Western blot analysis of **(C)** Tgl1p-V5 in *yeh1*Δ*yeh2*Δ was performed with total cell extracts from cells grown for times as indicated after addition of 100 µg/ml cycloheximide to cells grown to the mid logarithmic phase. The primary antibody was directed against the V5-tag (V5). The cytosolic marker protein glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as loading control. Western blot analyses shown are one representative experiment of at least three independent experiments. Relative amounts of **(D)** Tgl1p-V5 in wild type (WT) (black bar) and

*yeh1*Δ*yeh2*Δ (dark grey bar) obtained by three Western blots were calculated using the ImageJ software with the respective deviations as shown. The amounts of protein at time point 0 (addition of cycloheximide) were set at 1.

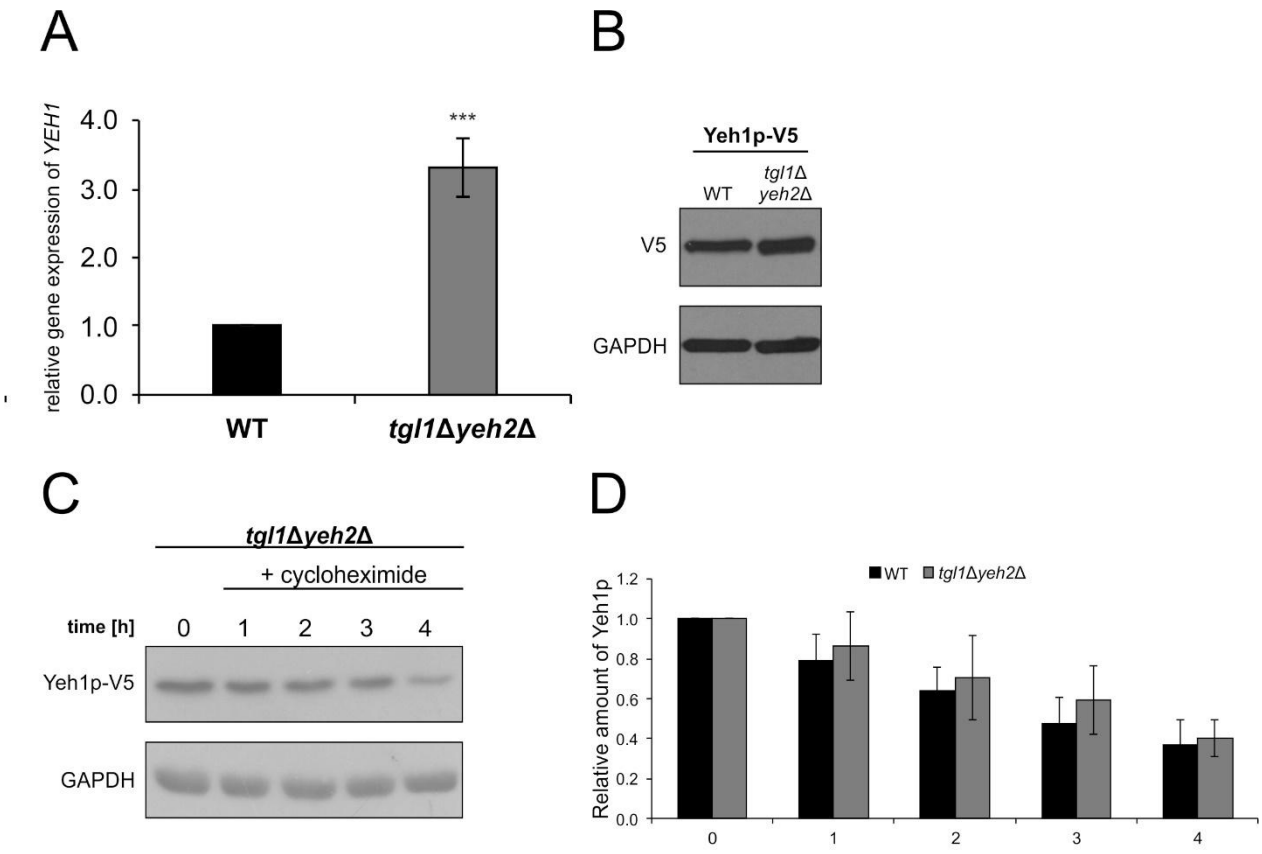

Figure 10 Gene expression, protein level and stability of Yeh1p in cells defective in both counterpart SE hydrolases.

Relative gene expression of **(A)** *YEH1* in wild type (WT) (black bar) and *tgl1*Δ*yeh2*Δ (dark grey bar) was measured by real-time PCR. The wild-type value was set at 1. Data are mean values from at least three independent experiments with the respective standard deviations as shown. Statistical significance was determined using Student's unpaired *t*-test, ***$p < 0.001$. **(B)** Protein analysis of V5-tagged Yeh1p from total cell extracts of wild type and *tgl1*Δ*yeh2*Δ grown to the mid logarithmic growth phase. The primary antibody was directed against the V5-tag (V5). The cytosolic marker protein glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as loading control. Western blot analysis shown is one representative of three independent experiments. To test protein stability of Yeh1p-V5 in the absence of both respective SE hydrolases Western blot analysis of **(C)** Yeh1p-V5 in *tgl1*Δ*yeh2*Δ was performed with total cell extracts from cells grown for times as indicated after addition of

100 µg/ml cycloheximide to cells grown to the mid logarithmic phase. The primary antibody was directed against the V5-tag (V5). The cytosolic marker protein glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as loading control. Western blot analyses shown are one representative experiment of at least three independent experiments. Relative amounts of **(D)** Yeh1p-V5 in wild type (WT) (black bar) and *tgl1*Δ*yeh2*Δ (dark grey bar) obtained by three Western blots were calculated using the ImageJ software with the respective deviations as shown. The amounts of protein at time point 0 (addition of cycloheximide) were set at 1.

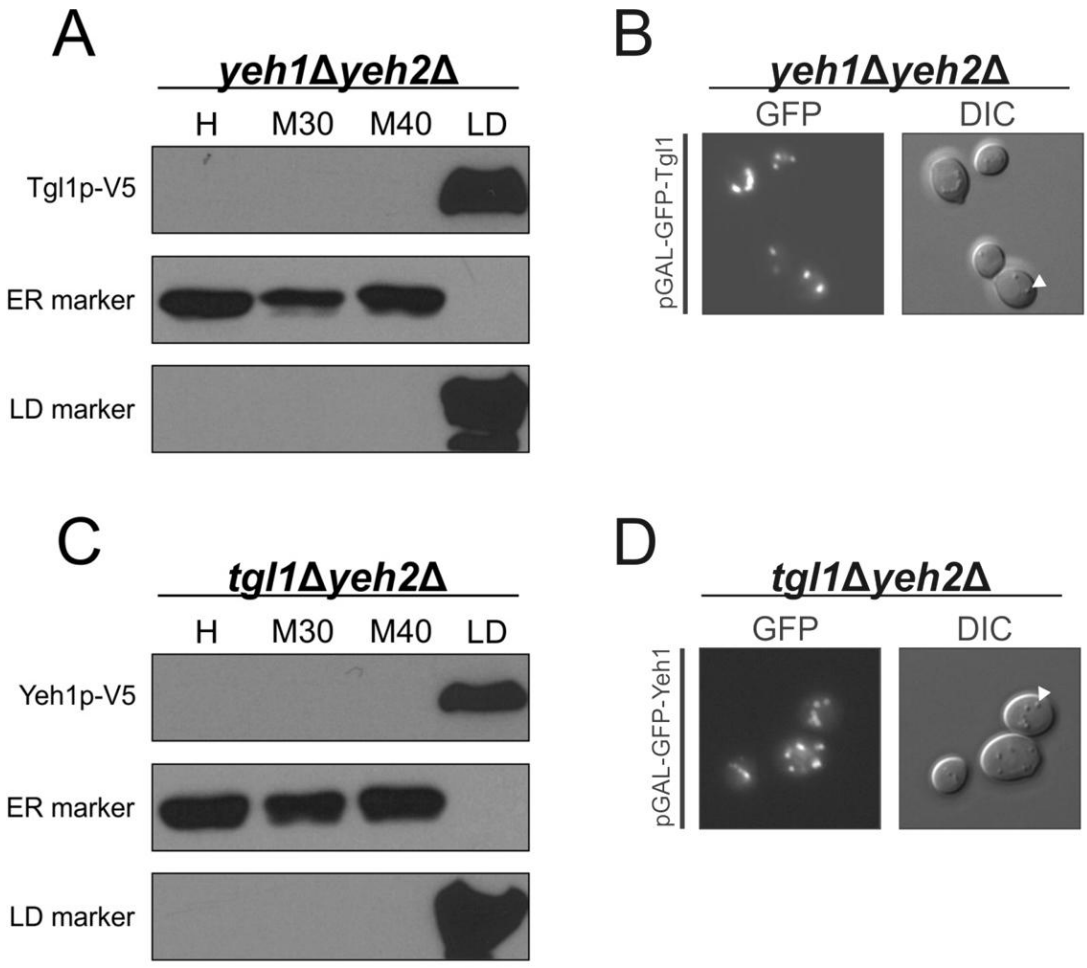

Figure 11 Localization of Tgl1p and Yeh1p in cells defective in both counterpart SE hydrolases.

Western blots of **(A)** Tgl1p-V5 in *yeh1*Δ*yeh2*Δ and **(C)** Yeh1p-V5 in *tgl1*Δ*yeh2*Δ cells grown to the stationary phase performed with homogenate (H), 30,000 x g microsomal (M30), 40,000 x g microsomal (M40) and lipid droplet (LD) fractions are shown. Primary antibodies were directed against the V5-tag (Tgl1p-V5 and Yeh1p-V5), Wbp1p or 40 kDa protein (ER

marker), and Erg6p or Ayr1p (LD marker). Western blot analyses shown are one representative of at least two independent experiments. Fluorescence microscopy was performed with N-terminally GFP-tagged variants of **(B)** Tgl1p in *yeh1*Δ*yeh2*Δ and **(D)** Yeh1p in *tgl1*Δ*yeh2*Δ cells using a galactose inducible promotor. DIC, differential interference contrast. Arrow heads are indicating LD.

4. Discussion

Tgl1p, Yeh1p and Yeh2p are the three major SE hydrolases in the yeast *S. cerevisiae*. In the past decades, several research groups unraveled interesting aspects concerning characterization of these SE hydrolases. Today it is known, that Tgl1p, Yeh1p, and Yeh2p differ in their *in vitro* activity, their contribution to *in vivo* SE mobilization under specific conditions and in their substrate specificities [22,23,26]. In terms of the regulation, Köffel and Schneiter [24] showed that the absence of oxygen mimicked by a heme deficiency mutant shifted the focus from Tgl1p to Yeh1p as the major SE hydrolase. Previous studies from our lab showed that deletion of the three SE hydrolases caused feedback regulation to the synthesis of nonpolar lipids [47]. In the *tgl1*Δ*yeh1*Δ*yeh2*Δ strain reduced activity of the SE synthesizing enzymes, Are1p and Are2p, and enhanced TG formation were observed.

To increase our knowledge about the SE metabolic network, we provide here some insight into regulatory aspects of the two LD localized SE hydrolases Tgl1p and Yeh1p in response to the presence or absence of nonpolar lipids. We investigated the gene expression level, the protein amount and the protein stability, as well as the localization of Tgl1p and Yeh1p in different strain backgrounds. One major finding of this work was the delocalization of Tgl1p and Yeh1p to the ER in the absence of both nonpolar lipids, TG and SE. This finding, however, was not surprising as the quadruple mutant *dga1*Δ*lro1*Δ*are1*Δ*are2*Δ (QM) completely lacks the host organelle of Tgl1p and Yeh1p, the LD. Previous studies already described the retention of typical LD proteins to the ER in the QM [28,29,45,46]. We also wanted to know whether the lack of SE alone, the substrate of SE hydrolases, was already sufficient to cause a changed localization of Tgl1p and Yeh1p to the ER. However, our experiments shown in Figure 4 B, Figure **5** B, Figure 6 and Figure 7 indicated clearly that Tgl1p as well as Yeh1p were still present exclusively in LD of an *are1*Δ*are2*Δ strain. This finding is in line with our previous observations made with TG lipases. These studies demonstrated that in the absence of their TG substrate the major amounts of Tgl3p, Tgl4p and

Tgl5p were still detectable in the LD fraction and only minor amounts of Tgl3p and Tgl5p shifted to the ER [28,29]. Such a shift was explained by the fewer and smaller LD in a *dga1*Δ*lro1*Δ strain [8] resulting in limited space for proteins on LD surface. Another explanation for the dual localization of the TG lipases might be their additional function in lipid anabolism. Studies from our laboratory demonstrated a lysophospholipid acyltransferase activity for Tgl3p, Tgl4p and Tgl5p [48,49]. In the case of Tgl3p, the lysophosphatidylethanolamine acyltransferase activity is favored, whereas Tgl4p and Tgl5p rather convert lysophosphatidic acid to phosphatidic acid in an acyl-CoA dependent manner. Moreover, one previous study revealed that the lipid composition of LD seems to influence the protein composition of the organelle [8], which might be relevant for the subcellular distribution of the TG lipases. Interestingly, the described shift of proteins in the *dga1*Δ*lro1*Δ strain to the ER was not observed with the two SE hydrolases. Neither for Tgl1p-V5 nor for Yeh1p-V5 a dual localization was observed in strains lacking TG or SE. One possible explanation might be the low abundance of Tgl1p-V5 and Yeh1p-V5 compared to the TG lipases, which makes it difficult to detect very small amounts of the proteins in the ER. However, we can summarize that the two LD resident SE hydrolases Tgl1p and Yeh1p behave like the TG lipase Tgl4p regarding their localization [29] and stay in their host organelle despite the absence of their substrate.

Another regulatory issue that we studied was the stability of Tgl1p-V5 and Yeh1p-V5. We found out that in the *dga1*Δ*lro1*Δ strain lacking TG, the stability of both SE hydrolases was markedly reduced, although the proteins were still localized to LD. In the QM where Tgl1p-V5 and Yeh1p-V5 were retained to the ER both proteins became even less stable than in the *dga1*Δ*lro1*Δ strain. Therefore, it seems that already the absence of TG and not only the localization renders the proteins instable.

A challenge for enzymes located to LD and the ER is how to deal with insertion into a phospholipid monolayer on the one hand, and into a bilayer on the other hand. As an example, Koch *et al.* [50] reported on the altered topology of Tgl3p depending on its environment. The authors found out that the stability of Tgl3p is regulated by its C-terminus. In the monolayer of LD, the C-terminus of Tgl3p is oriented towards inside the organelle, whereas in the bilayer of the ER it protrudes into the cytosol. Thus, Tgl3p localized in the ER becomes more prone to proteolytic digestion resulting in a reduced stability. As we do not have topology data of Tgl1p and Yeh1p we can only speculate that similar aspects may also

explain the instability of Tgl1p and Yeh1p in the QM (see Figure 2 D, E and Figure 3 D, E). Furthermore, it was surprising to see that both SE hydrolases were less stable in cells lacking TG (*dga1*Δ*lro1*Δ) compared to the *are1*Δ*are2*Δ strain which is unable to synthesize the substrate of both enzymes. A similar phenomenon was also observed for Tgl5p [29]. This TG lipase showed reduced stability in the *are1*Δ*are2*Δ strain, whereas the lack of TG did not influence the protein stability.

Beside the strongly compromised stability, both SE hydrolases Tgl1p and Yeh1p lost their capacity to hydrolyze SE when retained in the ER (see Figure 8A). In the absence of TG only, the protein stability of Tgl1p and Yeh1p was also strongly compromised (see Figures 2 and 3). However, under these conditions neither the intracellular localization nor the SE hydrolase capacity of Tgl1p and Yeh1p was affected (see Figures 4 to 8). These data suggest that Tgl1p and Yeh1p adopt a different topology in the bilayer membrane of the ER and the phospholipid monolayer covering LD, and as a consequence lose their enzyme activity.

Finally, we addressed the regulation of Tgl1p-V5 and Yeh1p-V5 among each other. As already described in the results, no major effects were observed with the exception of the upregulation of mRNA level for *YEH1* in the *tgl1*Δ*yeh2*Δ strain (see Figure 10 A). Yeh1p is the major SE hydrolase under anaerobic conditions [24]. In the *tgl1*Δ*yeh2*Δ strain, Yeh1p is the only SE hydrolase present. To compensate for the lack of the other two SE hydrolases, the cells might react with the observed upregulation of *YEH1* at mRNA level already under aerobiosis. However, the 3-fold upregulation of the mRNA level of *YEH1* is not reflected at protein level. In the *tgl1*Δ*yeh2*Δ strain the amount of Yeh1p-V5 is similar to wild type. Findings described herein suggest that Tgl1p and Yeh1p rather act independently of each other.

In summary, we provide here several pieces of evidence addressing the regulation of SE hydrolases in the yeast *Saccharomyces cerevisiae*. These novel findings contribute to our knowledge of nonpolar lipid metabolic network.

Acknowledgments

We thank the Austrian Centre of Industrial Biotechnology (ACIB) Graz for providing the ABI 7500 instrument. The contribution of Helmut Bergler in terms of providing the fluorescence

microscopic facility is gratefully acknowledged. This research was funded by the Austrian Science Fund (FWF) projects W901 DK Molecular Enzymology, P23029 to G. D. and P26308 to K. A..

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CHAPTER IV

Yeh2p – the cell periphery localized steryl ester hydrolase in the yeast *Saccharomyces cerevisiae*

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Preliminary manuscript

Abstract

In the yeast *Saccharomyces cerevisiae*, the three enzymes responsible for the hydrolysis of steryl esters are Tgl1p, Yeh1p and Yeh2p. One of the main differences between the three hydrolases is their intracellular localization. Tgl1p and Yeh1p are lipid droplet associated proteins, whereas Yeh2p is located at the cell periphery. In the present manuscript we confirmed the plasma membrane localization of Yeh2p and showed the contribution of Yeh2p to *in vivo* steryl ester mobilization. Furthermore, we showed that Yeh2p undergoes a posttranslational modification, namely phosphorylation. We also aimed to investigate the fate of Yeh2p in a strain lacking steryl esters, the substrate of Yeh2p, and in a strain lacking both major nonpolar lipids, steryl esters and triacylglycerols, and hence lipid droplets. Therefore, we analyzed the gene expression level, protein amount and localization of tagged variants of Yeh2p in respective deletion mutants. Under all tested conditions, the localization of Yeh2p was the plasma membrane. In strains lacking steryl esters or lipid droplets, the gene expression as well as the protein amount of Yeh2p was only slightly reduced compared to wild-type cells. In summary, we showed that the plasma membrane localized steryl ester hydrolase Yeh2p is modified posttranslationally and not regulated by the presence or absence of nonpolar lipids.

Highlights

- Yeh2p is one of three steryl ester hydrolases in *Saccharomyces cerevisiae*.
- Yeh2p is not localized to the lipid droplet, but to the plasma membrane.
- The protein undergoes a posttranslational modification, namely phosphorylation.
- The lack of steryl esters, the substrate of Yeh2p, as well as the lack of lipid droplets does not influence the localization of Yeh2p.

Key words: yeast, lipids, steryl ester(s), sterols, hydrolase, plasma membrane

Abbreviations: ER, endoplasmic reticulum; PM, plasma membrane; QM, quadruple mutant; SE, steryl ester; TG, triacylglycerol.

1. Introduction

One of the major sources of energy are fatty acids. Usually, fatty acids do not occur in their free form but as part of more complex lipids. Three fatty acids can be linked to a glycerol backbone forming triacylglycerols (TG). Another possibility to store fatty acids is the esterification of sterols at the 3' hydroxy group. The resulting steryl esters (SE) and TG are the most prominent nonpolar lipids synthesized in the endoplasmic reticulum (ER) and stored in an intracellular organelle called lipid droplet (LD) [1,2]. These esterification reactions are crucial to keep lipid homeostasis in living cells balanced.

In our model system, the yeast *Saccharomyces cerevisiae*, the ratio between SE and TG in LD is almost 1:1 [3–5]. While the two acyl-CoA:sterol acyltransferases Are1p and Are2p (ACAT related enzymes) are responsible for the formation of SE [6,7], Dga1p, a diacylglycerol:acyl-CoA acyltransferase, and Lro1p, a phospholipid:diacylglycerol acyltransferase, catalyze the synthesis of TG [8–11]. A yeast mutant strain lacking both SE synthesizing enzymes, Are1p and Are2p, is devoid of SE but still forms TG and hence LD. In contrast, the quadruple mutant *dga1*Δ*lro1*Δ*are1*Δ*are2*Δ (QM) harbors none of the enzymes responsible for the formation of TG and SE and consequently lacks LD [12].

Both nonpolar lipids, SE and TG, accumulate during the late exponential growth phase and are stored in the hydrophobic core of LD [4]. The LD is enwrapped by a phospholipid monolayer with several proteins embedded [3–5]. Examples for LD associated proteins are the TG lipases or two of the SE hydrolases, namely Tgl1p and Yeh1p. Upon requirement, the two LD associated SE hydrolases can cleave SE and set free sterols and a fatty acid. Both enzymes show a low *in vitro* SE hydrolase activity and were identified as paralogues of mammalian acid lipases [13]. However, the third and main SE hydrolase under aerobic conditions is Yeh2p, an enzyme surprisingly not localized to LD but to the cell periphery [13,14].

Although there is evidence for a plasma membrane (PM) localization of Yeh2p [14], some doubts about this subcellular localization were presented and Yeh2p was ascribed to the ER. In fact, 20-45% of the PM in yeast cells have an underlying network of peripheral ER, with an average distance of 30 nm between these organelles [15,16]. Places where membranes of two organelles are in juxtaposition with each other are called membrane contact sites (MCS) [17–19]. These sites are important for calcium transport [20] and lipid signaling, especially nonvesicular sterol lipid transport [19,21] in eukaryotic cells. In yeast cells, three conserved protein families facilitate the ER-PM tether [22–25], namely the yeast VAP

(VAMP (vesicle associated membrane protein)-associated proteins) proteins Scs2p, Scs22p, and Ist2p (related to the TMEM16 channel family [26,27]), and the tricalbin proteins Tcb1p, Tcb2p and Tcb3p (orthologs of the extended synaptotagmin-like proteins E-Syt 1/2/3 [28]). Upon loss of all six ER tether proteins, the morphology of the cortical ER is drastically altered. Wild-type (WT) tether cells with a functional ER-PM tether show a network of sheets and tubes of the ER attached to the PM. Delta tether strains, however, have a massive reduction in ER-PM contacts down to approximately 5% and accumulate ER in the cytoplasm [23,29,30].

In the present study we aimed to clarify the subcellular localization of Yeh2p. For this reason we performed Western blot analysis with highly purified cell fractions from WT tether and Delta tether strains, and additionally analyzed the localization of GFP-tagged Yeh2p by fluorescence microscopy. Furthermore, we studied the contribution of Yeh2p to *in vivo* SE mobilization and detected a posttranslational modification of Yeh2p. Since recent studies from our laboratory revealed regulatory effects of the presence or absence of nonpolar lipids on the two LD resident SE hydrolases Tgl1p and Yeh1p [31] as well as on the TG lipases [32,33], we extended these studies to Yeh2p. Therefore, we investigated the transcription and protein level as well as the subcellular localization of Yeh2p in the absence of SE or LD. In summary, we could demonstrate that Yeh2p is a phosphorylated protein localized at the PM which seems to be unaffected by the presence or absence of SE or LD.

2. Materials and Methods

2.1. Yeast strains and culture conditions

Yeast strains used in this study are listed in Table 1. Yeast cells were either grown in YPD medium containing 1% yeast extract (Oxoid Ltd, Basingstoke, UK), 2% glucose (Roth, Karlsruhe, Germany) and 2% peptone (Oxoid Ltd); or in synthetic minimal medium (SD) containing 0.67% yeast nitrogen base (ForMedium, Hundstanton, UK), 2% glucose and the respective amino acid supplements. Cells were cultivated in liquid medium at 30°C under vigorous shaking either to the exponential or to the early stationary phase. Gal1 promoter-controlled genes were induced by growing cells in synthetic minimal medium containing 2% galactose (ForMedium) as a carbon source. Growth was monitored by measuring the optical density at 600 nm (A_{600}).

Table 1 Yeast strains used throughout this study.

Strain	Genotype	Source
Wild type (WT)	BY4741 Mat a; *his3Δ1; leu2Δ0; met15Δ0; ura3Δ0*	Euroscarf
GFP-Yeh2p	BY4741; *HIS3MX6::PGAL1-GFP* (S65T)-*YEH2*	This study
Yeh2p-V5	BY4741*; YEH2-V5::HIS3MX6*	This study
Yeh2p-GFP	BY4741*; YEH2-GFP::HIS3MX6*	This study
are1Δare2Δ	BY4742; *are1Δ::kanMX4; are2Δ::URA3*	Kindly provided by M. Spanova
are1Δare2Δ GFP-Yeh2p	*are1Δare2Δ; HIS3MX6::PGAL1-GFP* (S65T)-*YEH2*	This study
are1Δare2Δ Yeh2p-V5	*are1Δare2Δ; YEH2-V5::HIS3MX6*	This study
QM	BY4741; *dga1Δ::kanMX4; lro1Δ::kanMX4; are1Δ::kanMX4; are2Δ::kanMX4*	Athenstaedt [34]
QM GFP-Yeh2p	QM; *HIS3MX6::PGAL1-GFP* (S65T)-*YEH2*	This study
QM Yeh2p-V5	QM, *YEH2-V5::HIS3MX6*	This study
tgl1Δyeh1Δ	BY4741; *tgl1Δ::kanMX4; yeh1Δ::kanMX4*	Wagner *et al.* [35]
tgl1Δyeh1Δ Yeh2p-GFP	*tgl1Δyeh1Δ; YEH2-GFP::HIS3MX6*	This study
tgl1Δyeh1Δyeh2Δ	BY4741; *tgl1Δ::kanMX4; yeh1Δ::kanMX4; yeh2Δ::kanMX4*	Wagner *et al.* [35]
WT tether	SEY6210.1 Mat a; *ura3-52, his3-Δ200, trp1-Δ901, lys2-801, suc2-Δ9, leu2-3112*	A gift from S. Emr to R. Schneiter
WT tether Yeh2p-GFP	WT tether; *YEH2-GFP::URA3*	This study
WT tether mCherry- HDEL	WT tether; p mCherry-HDEL	This study
WT tether Pma1-RFP	WT tether; pRS416 ADH1 Pma1-mRFP	This study
Delta tether	SEY6210.1 Mat a; *ist2Δ::HIS3MX6; scs2Δ::TRP1; scs22Δ::HIS3MX6; tcb1Δ::kanMX6; tcb2Δ::kanMX6, tcb3Δ::HIS3MX6*	A gift from S. Emr to R. Schneiter
Delta tether Yeh2p-GFP	Delta tether; *YEH2-GFP::URA3*	This study
Delta tether mCherry- HDEL	Delta tether; p mCherry-HDEL	This study
Delta tether Pma1-RFP	Delta tether; pRS416 ADH1 Pma1-mRFP	This study

2.2. Genetic techniques

Chromosomal tagging of *YEH2* was performed by homologous recombination using the PCR-mediated method of Longtine *et al.* [36]. In brief, the inserts for the construction of the Yeh2p-V5 and pGAL1-GFP-Yeh2p strains were obtained by PCR using genomic DNA from a strain bearing a 6xGLY-V5-HIS3MX6 construct and the plasmid pFA6a-HIS3MX6-PGAL1-GFP(S65T), respectively, as a template. The insert for chromosomal GFP-tagging of Yeh2p in the WT tether and the Delta tether strains was amplified from the plasmid pKT209 (pFA6a-link-yEGFP-*CaURA3*; Thorn *et al.* [37]). Primers used for amplification of the respective DNA-fragments are listed in Table 2. 500 ng insert DNA were used for transformation of yeast strains employing the high-efficiency lithium acetate transformation protocol [38]. After transformation, cells were plated on minimal medium lacking histidine or uracil for selection and incubated for 2 to 3 days at 30°C. Positive transformants were verified by colony PCR of whole yeast cell extracts with primers listed in Table 2.

The plasmid containing mCherry fused to the ER retention signal HDEL was a gift from the Schneiter lab. For the construction of the Pma1-RFP plasmid, the plasmid VTU100_Pma1-mRFP (kindly provided by Vendula Stradalova) was used as a template to amplify the DNA encoding Pma1-mRFP with primers listed in Table 2. Next, the Pma1-mRFP insert was integrated into the plasmid pRS415 ADH1 (kindly provided by the Schneiter lab) via homologous recombination using the lithium acetate method [38]. 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 by colony PCR with primers listed in Table 2 and by fluorescence microscopy.

Table 2 Primers used throughout this study.

Primer	Sequence (5’ → 3’)
Fwd_Yeh2_C_tag_GFP	ATAGAATTGGTAAGCCAATGATAGAAAATTTGAGGTTTCCT AATGCAAGGCGGATCCCCGGGTTAATTAA
Rev_Yeh2_C_tag_GFP	TTATGGCATATTATATTTTACAAAGAAACCACAAAGAAAAA ACTTTTACCGAATTCGAGCTCGTTTAAAC
Yeh2contr_fwd	ACACATTATAATGTTTAT
GFP_rev_contr	GTAGTTTTCCAGTAGTGC
Fwd_Yeh2_V5tag_Cterm	ATAGAATTGGTAAGCCAATGATAGAAAATTTGAGGTTTCCT AATGCAAGGAAGGGCGAGCTTCGAGGTCA

Fwd_Yeh2_Ntag_GFP	AATTGTTGGGAAATACTCAACTATCATTGGGATCCCCGTGGC ACCTTTACGATAAGCGAATTCGAGCTCGTTTAAAC
Rev_Yeh2_Ntag_GFP	AAACGAAGTGAGAATTATTGCGCTCACCAACCGCTGAACCT CATCAACCACCTTATTTACTTTGTATAGTTCATCCATGC
Contr_N-term_GFP_fwd	CATTCTTGGACA
Contr_Yeh2+200	GTCTAGAGTGAC
Fw_Pma1RFP_FriCl	CTAGTGGATCCCCCGGGCTGCAGGAATTCGATATCAATGAC TGATACATCATCCTC
Rev_Pma1RFP_FriCl	GAAAAGGGGCCTGTCTCGAGGTCGACGGTATCGATAAGCTT TAGGCGCCGGTGGAGTG
Rev_GFP_Thorn	ATGGAACTGGCAATTTACCAG
S150_Rev_GFP Sté	AGACAACCATTACTTATC
Fw_pKT209_GFP_ura	GTAAACCAATGATAGAAAATTTGAGGTTTCCTAATGCAAGG GGTGACGGTGCTGGTTTA
Rev_pKT209_GFP_ura	CATATTATATTTTACAAAGAAACCACAAAGAAAAAACTTTT ACCTCGATGAATTCGAGCTCG
ADH fw	GTCGTTGTTCCAGAGCTGAT
Pma1h rev	GCAAGAAACAGTCTTCAGTG

2.3. RNA isolation and real-time PCR

Total RNA isolation from cells grown to the mid logarithmic phase was performed as described by the manufacturer using the RNeasy kit from Qiagen (Hilden, Germany). After DNaseI digestion, quantitative real-time PCR was performed using SuperScript III Platinum SYBR Green One-Step qRT-PCR Kit (Invitrogen) according to the manufacturer's instructions. Reactions were performed in sealed MicroAmp Optical 96-Well Reaction Plates, and amplification was measured using an ABI 7500 instrument (Applied Biosystems). Samples were quantified using the $\Delta\Delta C_t$ method described by Livak and Schmittgen [39]. *ACT1* served as internal control. Primers used for real time-PCR are listed in Table 3.

Table 3 Primers used for qRT-PCR.

Primer	Sequence (5' → 3')
YEH2_RTFw	GCAATGGTGGTTGTCACCTCTTCCTAATAAG
YEH2_RTRev	CTGTCATCATCATTCTTGGCAATAGGAAACCATG

ACT1_RTFw	CCAGCCTTCTACGTTTCCATCCAAG
ACT1_RTRev	GACGTGAGTAACACCATCACCGGA

2.4. Isolation and characterization of the plasma membrane and microsomes

Yeast cells were grown aerobically in YPD to the early stationary phase at 30°C. Cells were disrupted in the presence of glass beads using a Merckenschlager homogenizer under CO_2-cooling. In order to remove the glass beads, cell debris and unbroken cells, the cell extract was centrifuged at 2500 x g for 5 min. The supernatant yields the homogenate. Crude plasma membrane was isolated as described by Serrano [40] and further purified according to van den Hazel *et al.* [41] and Pichler *et al.* [42]. Isolation of highly pure microsomes was performed as described previously [43–45]. The protein concentration was analyzed by the method of Lowry *et al.* [46] using bovine serum albumin as a standard. Proteins were precipitated with trichloroacetic acid at a final concentration of 10% and solubilized in 0.1% SDS, 0.1% NaOH. Proteins were separated by SDS-PAGE using 10% polyacrylamide gels according to the method of Laemmli [47]. Western blot analysis was performed as described by Haid and Suissa [48]. 10 µg of proteins from each fraction were loaded, and proteins were detected using mouse or rabbit antisera as primary antibody. The antibodies used were directed either against the GFP-tag (Roche) or against the V5-tag (Invitrogen); Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) or Pgk1p (cytosolic marker); Wbp1p or 40 kDa protein (ER-marker); and Pma1p, the plasma membrane P2-type H+-ATPase (plasma membrane marker). Primary antibodies were detected with peroxidase-conjugated goat anti-mouse or anti-rabbit IgG, respectively, as second antibody and SuperSignal® West Pico Chemiluminescent substrate solution (Thermo Scientific, Rockford, Illinois, USA) or Amersham™ ECL Select™ Western blotting Detection Reagent (GE Healthcare, Buckinghamshire, UK).

2.5. *In vivo* steryl ester mobilization assay

The *in vivo* SE mobilization assay was performed as described in Köffel *et al.* [13]. To label nonpolar lipids, cells were incubated with 10 µCi of [^{3}H] palmitic acid (American Radiolabeled Chemicals Inc., St. Louis, MO.) per mL for 16 h at 30°C. After a washing step, cells were diluted in YPD medium containing 30 µg terbinafine (a kind gift from N. Ryder (Novartis Research Institute, Vienna, Austria) to the Schneiter lab) per mL. At time points

indicated, aliquots of cultured cells were removed. After two freeze/thawing cycles in liquid nitrogen, cells were lysed in the presence of glass beads by thorough vortexing. Lipids were extracted with chloroform/methanol (1:1, v/v). Radioactivity was measured by liquid scintillation counting. Sample volumes of equal counts were brought to dryness, dissolved in a small volume of chloroform/methanol (1:1, v/v) and spotted onto thin-layer chromatography (TLC) plates (Merck, Darmstadt, Germany). Nonpolar lipids were separated by using the solvent system light petroleum/diethyl ether/acetic acid (70/30/2, per vol.). For quantification of the radiolabel in SE, a Berthold Tracemaster 40 Automatic TLC-Linear Analyzer was used.

2.6. Phosphatase Assay

Cells were cultured in YPD to the early logarithmic growth phase. 30 OD units of the culture were harvested and diluted in trichloroacetic acid at a final concentration of 10%. After a 30 min incubation step on ice, cells were centrifuged and washed twice with ice cold water. The cell pellet was resuspended in buffer A (1% SDS, 0.1 M Tris/HCl pH 8, 1% β-mercaptoethanol) and vortexed for 10 min in the presence of glass beads. After a 5 min heating step at 95°C, samples were diluted with buffer B (50 mM Tris/HCl pH 8, 1 mM $MgCl_2$, 0.5% Triton X-100) and centrifuged at 4°C for 5 sec at full speed. Then the supernatant was aliquoted to three new Eppendorf tubes. In one tube, 800 units of lambda protein phosphatase (New England BioLabs® Inc., Frankfurt am Main, Germany) were added. In a second tube, 800 units of heat inactivated lambda protein phosphatase (heated at 95°C for 1 hour in the presence of 50 mM Na_2EDTA) were added. The third aliquot was used as a negative control lacking any protein phosphatase. After a 1 hour incubation step at 30°C proteins were precipitated with trichloroacetic acid at a final concentration of 10%. Proteins were separated by SDS-PAGE using 10% polyacrylamide gels according to the method of Laemmli [47]. Western blot analysis was performed as described above. In case of the concentration dependent phosphatase experiment 0.5 µL, 1 µL, 2 µL, 3 µL, 4 µL, 5 µL and 10 µL of alkaline phosphatase (from bovine intestinal mucosa, 18312 U/mL; Sigma-Aldrich, St. Louis, MO) were used instead of the lambda protein phosphatase.

2.7. Fluorescence microscopy

Yeast strains were grown at 30°C in synthetic minimal medium (SD) (see Section 2.1) to the late logarithmic growth phase. To induce the expression of recombinant GFP-

Yeh2p under control of the Gal1 promoter in WT, *are1*Δ*are2*Δ and the QM, an aliquot of the culture was shifted to galactose containing SD medium for 6-8 h. Fluorescence microscopy was carried out on a Zeiss Axioskop microscope using a ×100 oil immersion objective with a narrow band enhanced GFP (eGFP) filter (Zeiss). Images were taken with a Visicam CCD camera and displayed using the Metamorph Imaging software (Visitron Systems, Puchheim, Germany). Transmission images were obtained by using Nomarski optics (differential interference contrast). For visualization of C-terminally GFP-tagged Yeh2p expressed from its endogenous promotor in the WT tether and the Delta tether strains, the inverted Spinning Disk Confocal Microscope VisiScope CSU-W1 (Bioimage, Light Microscopy Facility – Departments of Biology and Medicine, University of Fribourg) with a 50 µm pinhole disk, and the scientific grade 4.2 sCMOS camera were used.

3. Results

3.1. Localization of Yeh2p

Although there is general agreement that Yeh2p is a protein resident in the cell periphery, either the plasma membrane (PM) or the peripheral/cortical endoplasmic reticulum (cER) are believed to be the intracellular localization of Yeh2p. The reason for the controversial opinions about the intracellular localization of Yeh2p is the close contact of the cER and PM. These membrane contacts depend on tether proteins. A Delta tether strain lacking 6 tether proteins (*ist2*Δ*scs2*Δ*scs22*Δ*tcb1*Δ*tcb2*Δ*tcb3*Δ) shows a collapsed cER which is no longer associated with the PM. Thus, localization studies of a protein in the Delta tether strain background allow to clearly distinguish between a localization at the PM or the cER.

To determine the exact intracellular localization of Yeh2p we first tagged this SE hydrolase chromosomally with GFP in the Delta tether strain and the corresponding wild-type strain (WT tether). After isolation of the PM and the M30 and M40 microsomes by differential centrifugation, we analyzed these fractions by Western blotting. The signal for Yeh2p-GFP was most pronounced in the PM fraction of both the WT tether as well as the Delta tether strain (Figure 1). We also observed a very faint signal from Yeh2p-GFP in the M30 and M40 fractions of these strains. However, as the PM marker Pma1p was also slightly detectable in the M30 and M40 fractions, the faint signal of Yeh2p-GFP in microsomes of the Delta tether strain and control can be ascribed to a minor cross-contamination of the isolated

ER fractions with PM. Therefore, these results strongly indicate that Yeh2p is localized to the PM and not the cortical ER.

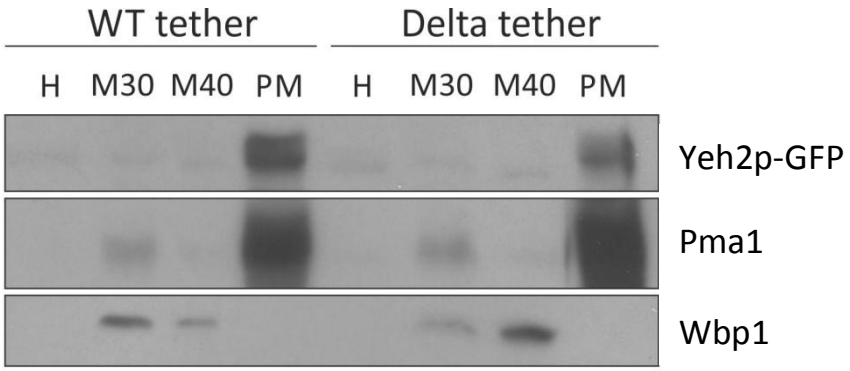


Figure 1 Localization of Yeh2p-GFP in cells with (WT tether) or without (Delta tether) the tether between the cortical endoplasmic reticulum and the plasma membrane.
Western blot analysis of Yeh2p-GFP was performed with homogenate (H), 30,000 x g microsomal (M30), 40,000 x g microsomal (M40) and the plasma membrane (PM) fraction from the WT tether and the Delta tether strain grown to the early stationary growth phase. Primary antibodies were directed against the GFP-tag (Yeh2p-GFP), Pma1p (PM marker) and Wbp1p (ER marker). Western blot analyses are representative of at least two independent experiments.

To further support our findings from the Western blot experiments, we also determined the localization of Yeh2p-GFP in the Delta tether and WT tether strains by fluorescence microscopy. For this approach, we transformed these strains additionally with a plasmid expressing either a fluorescently labelled ER marker (mCherry-HDEL) or a PM marker (Pma1p-RFP) (see Methods and Materials) to distinguish visually between the cER and PM. As shown in Figure 2A, with the PM marker Pma1p-RFP we observed a red ring surrounding the cells of both the Delta tether and the WT tether strains. The signal arising from the ER marker mCherry-HDEL which located at the cER was similarly observed as a red ring at the cell periphery of WT tether cells (Figure 2B, upper panel). In contrast, the Delta tether strain with its collapsed cER lacked the peripheral ER stain and showed dotted structures in the cytoplasm instead (Figure 2B, lower panel). The green signal arising from Yeh2p-GFP, however, appeared exclusively as dotted line at the cell periphery irrespective of the strain background (Figure 2 A and B). As in the Delta tether strain this green signal

matched exclusively with the red signal of the PM stain (Figure 2A), but not the ER marker (Figure 2B), these data clearly demonstrated that Yeh2p is indeed a PM resident protein.

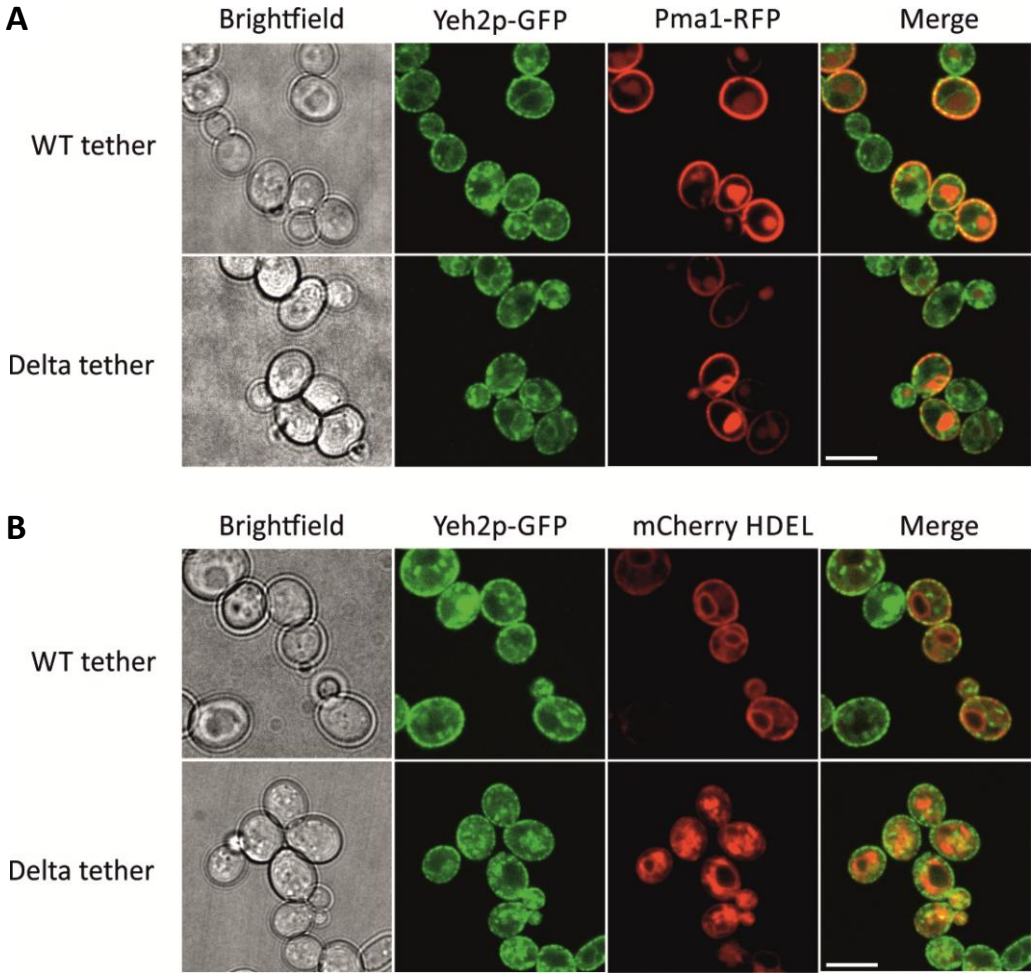


Figure 2 Fluorescence microscopy of GFP-tagged variants of Yeh2p in cells with (WT tether) or without (Delta tether) the tether between the cortical ER and the PM.
Fluorescence microscopy of WT tether and Delta tether with a genomically GFP-tagged variant of Yeh2p containing **(A)** the plasmid pRS416 ADH1 Pma1mRFP to highlight the PM and **(B)** the plasmid p mCherry-HDEL to highlight the ER. Cells were grown to the mid logarithmic growth phase. Scale bar: 5 µm.

3.2. The contribution of Yeh2p to *in vivo* steryl ester mobilization

To monitor the contribution of Yeh2p to SE mobilization *in vivo*, we performed SE mobilization assays tracing the amount of [^{3}H]palmitic acid labelled SE in different strain backgrounds over the time. We used the drug terbinafine, an inhibitor of fungal squalene

epoxidase to forces the cells to use up their SE stores for ongoing membrane formation. As a positive control, we used the WT strain containing all the three SE hydrolases in active form. As expected and already shown by Köffel *et al.* [13] we observed a very efficient *in vivo* SE mobilization for the WT, irrespective whether Yeh2p was GFP-tagged (Figure 3, white squares) or not (Figure 3 black squares). Both strains behaved the same; and 12 hours after addition of terbinafine, the level of [^{3}H]palmitic acid in SE was reduced to about 20% compared to time point zero. Hence, we could exclude an influence of the GFP-tag to the SE mobilization ability of Yeh2p. As a negative control we used the strain deleted in all three SE hydrolases *tgl1*Δ*yeh1*Δ*yeh2*Δ. This strain is unable to mobilize SE, which could be seen in Figure 3 (black diamonds). In a *tgl1*Δ*yeh1*Δ mutant, Yeh2p is the only SE hydrolase which can perform SE mobilization. As a proof of Yeh2p being indeed competent to mobilize SE, we could argue with the reduced radioactive label in SE in such a strain to about 70% at 12 hours after terbinafine addition (see black circles for the respective strain without the GFP-tag, or white circles with Yeh2p-GFP tagged). With these assays we confirmed the ability of Yeh2p to mobilize SE *in vivo*, and ascertained that the GFP-tag did not influence its activity.

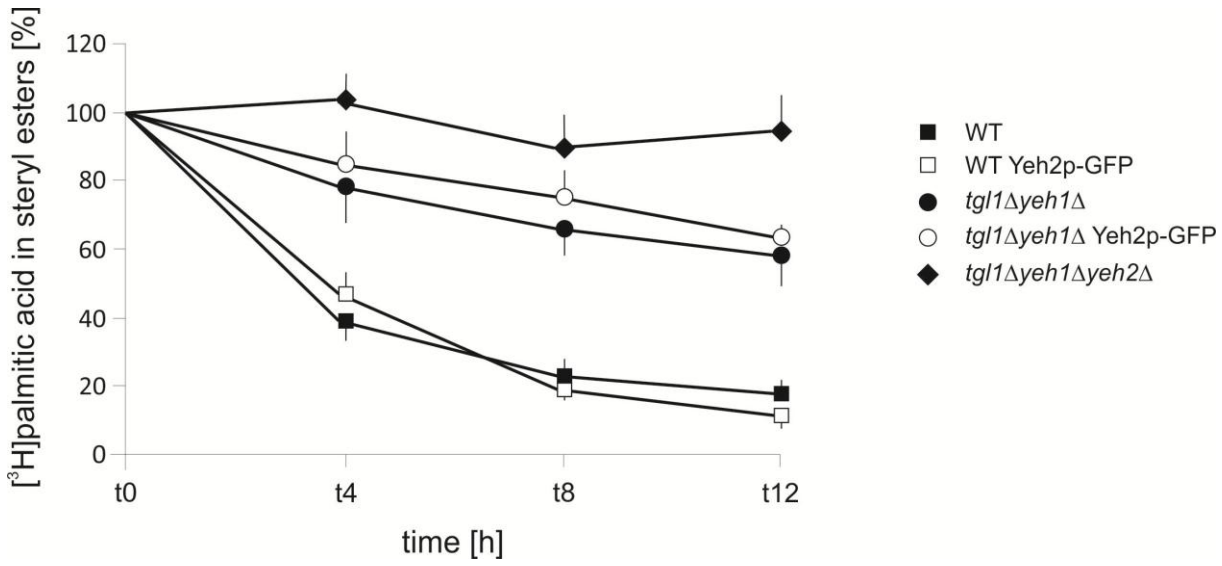


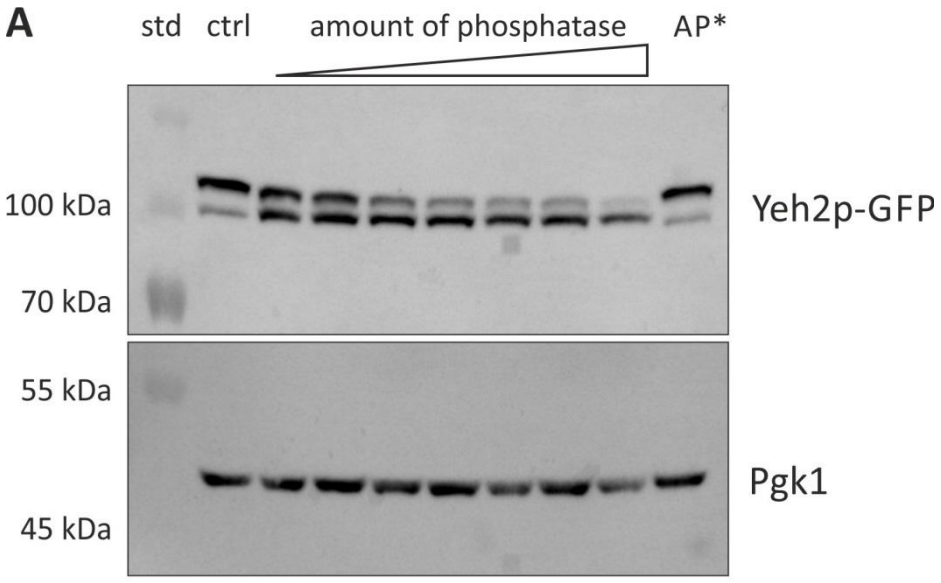
Figure 3 *In vivo* steryl ester mobilization of Yeh2p.

SE from WT (■), WT Yeh2p-GFP (□), *tgl1*Δ*yeh1*Δ (●), *tgl1*Δ*yeh1*Δ Yeh2p-GFP (○) and *tgl1*Δ*yeh1*Δ*yeh2*Δ (◆) were labeled for 16 h with [^{3}H]palmitic acid. Then, the *in vivo* SE mobilization was analyzed by determining the SE level at 0, 4, 8 and 12 h after dilution of the cells in medium containing terbinafine. Lipids were extracted and analyzed by TLC as described in Materials and Methods. The content of [^{3}H]palmitic acid in SE was quantified by radioscanning of TLC plates. WT values at time point 0 were set to 100%. Data are mean

values from at least two independent experiments in double estimation with the respective standard deviations as shown.

3.3. Yeh2p underlies a posttranslational modification, namely phosphorylation

The fact that we always saw a double band when we analyzed Yeh2p by Western blotting tempted us to speculate about a possible posttranslational modification of Yeh2p. One possibility is that the upper band of Yeh2p-GFP in wild-type cells might be a phosphorylated version of Yeh2p-GFP (see Figure 4A ctrl). For that reason we performed phosphatase assays as described in Materials and Methods. In Figure 4A we could see that the upper band of Yeh2p-GFP got less upon phosphatase treatment, indicating that Yeh2p is indeed a phosphorylated protein. As a control, we treated one aliquot of our sample with heat inactivated phosphatase. In this assay, the effect described above was not seen (see Figure 4A AP* and 4B third lane). The same observation was made when we replaced the GFP-tag by a very small tag, namely the V5-tag (see Figure 4B). Again, in the sample treated with phosphatase, the very upper band vanished and at the same time the signal from the lower band increased. Most interestingly, with the Yeh2p-V5 construct we could even detect a third, albeit very faint band in the middle of the outer ones, giving a hint to some more/other modifications going on.



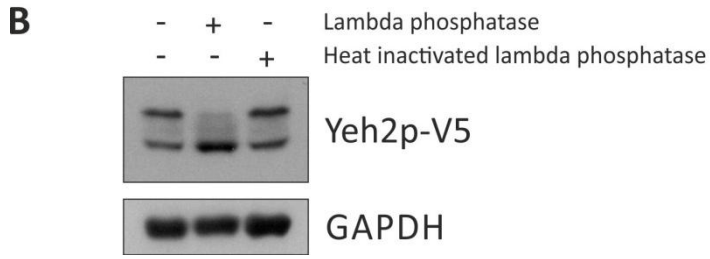


Figure 4 Western Blots demonstrated that Yeh2p is a phosphorylated protein.
(A) Cells from WT Yeh2p-GFP were treated with alkaline phosphatase as described in Materials and Methods. The sample in the first lane is the prestained protein standard ladder (std), the second lane represents WT Yeh2p-GFP without any phosphatase as a control sample (ctrl). Then, from left to the right, different amounts of alkaline phosphatase were used to treat the samples (from 0.5 µL up to 10 µL of 18312 U/mL alkaline phosphatase). The sample in the last lane was incubated with heat inactivated alkaline phosphatase (AP*). Primary antibodies were directed against the GFP-tag (Yeh2p-GFP), and the cytosolic marker protein phosphoglycerate kinase 1 (Pgk1p) which was used as loading control. Western blot analyses are representative of at least two independent experiments. **(B)** Cells from WT Yeh2p-V5 were either not treated with any phosphatase (lane 1), treated with lambda phosphatase (lane 2) or with heat inactivated lambda phosphatase (lane 3) as described in Materials and Methods. Primary antibodies were directed against the V5-tag (Yeh2p-V5) and the cytosolic marker protein glyceraldehyde-3-phosphate dehydrogenase (GAPDH) which was used as loading control. Western blot analyses are representative of at least three independent experiments.

3.4. Transcription level, protein level and localization of Yeh2p in cells lacking either steryl esters or both nonpolar lipid species

To test the influence of the absence of SE, the substrate of Yeh2p, or the lack of both major nonpolar lipids, SE and TG, and hence LD, we determined gene expression, protein level and localization of Yeh2p in respective strain backgrounds. An *are1*Δ*are2*Δ strain lacks both enzymes responsible for SE formation and therefore also SE. The quadruple mutant *dga1*Δ*lro1*Δ*are1*Δ*are2*Δ (QM), in contrast, is devoid of SE and TG and, as a consequence, lacks LD. Comparing the gene expression level of *YEH2* we could see a slightly although not significantly reduced mRNA level of *YEH2* in both mutant strains compared to wild type

(Figure 5A). Similar observations were made with the protein level. In Figure 5B the amounts of genomically V5-tagged variants of Yeh2p are shown. In the *are1*Δ*are2*Δ strain as well as in the QM the amounts of Yeh2p-V5 were the same, however, in wild-type cells there was slightly more protein present.

We also wanted to test whether the subcellular localization of Yeh2p was changed in the absence or presence of SE or LD. Fluorescence microscopy demonstrated that under both condition Yeh2p was still present in the PM (Figure 5C). In contrast, both LD resident SE hydrolases Tgl1p and Yeh1p [31], were shifted to the ER in the QM which lacks LD.

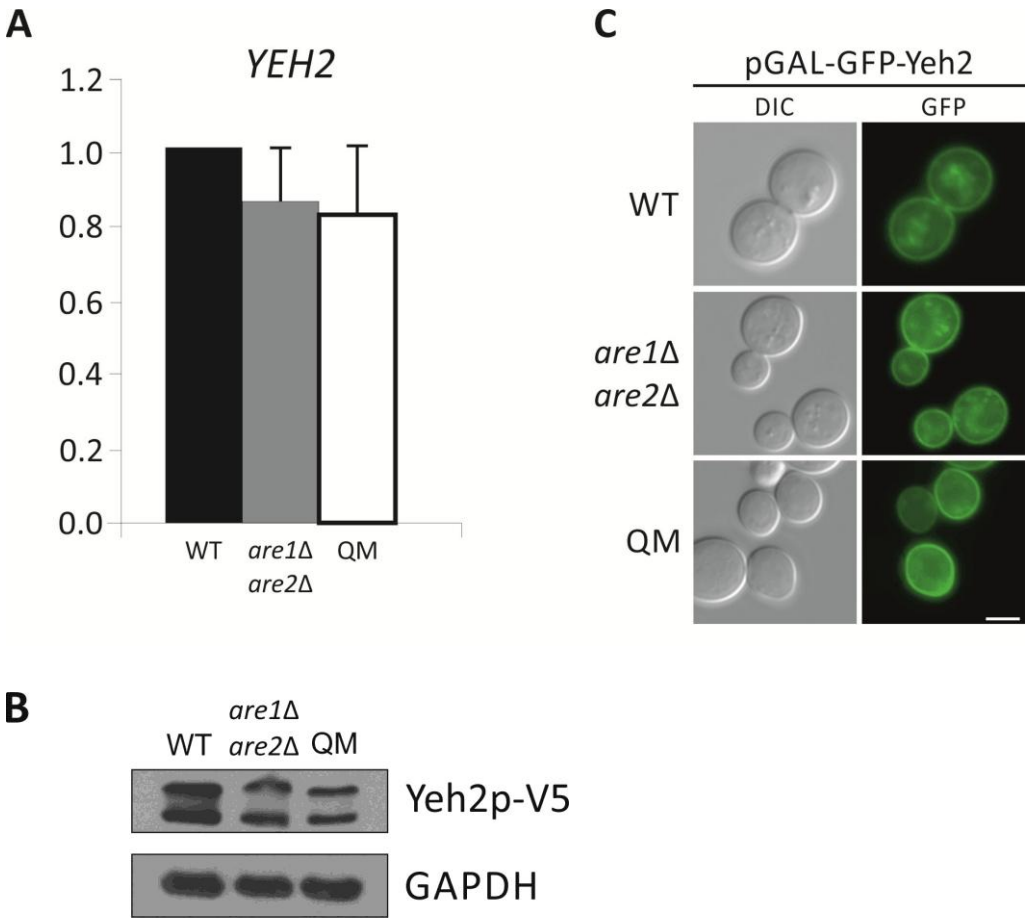


Figure 5 Gene expression, protein level and localization of Yeh2p in cells defective in nonpolar lipid synthesis.

(A) The relative gene expression of *YEH2* in wild type (WT) (black bar), *are1*Δ*are2*Δ (grey bar) and *dga1*Δ*lro1*Δ*are1*Δ*are2*Δ (QM) (white bar) strains was quantified by real-time PCR. Wild-type values were set at 1. Data are mean values from at least three independent experiments with the respective standard deviations. **(B)** Protein analysis of V5-tagged variants of Yeh2p from total cell extracts of wild type, *are1*Δ*are2*Δ and QM strains grown to

the mid logarithmic growth phase is shown. The primary antibody was directed against the V5-tag (V5). The cytosolic marker protein glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as loading control. **(C)** Fluorescence microscopy of GFP-tagged variants of Yeh2p. Fluorescence microscopy of pGAL1-GFP-Yeh2p in wild type (WT), *are1*Δ*are2*Δ and *dga1*Δ*lro1*Δ*are1*Δ*are2*Δ (QM) grown to the mid logarithmic growth phase. DIC, differential interference contrast. Scale bar: 5 µm.

4. Discussion

Previous studies of the three SE hydrolases Tgl1p, Yeh1p and Yeh2p in the yeast *Saccharomyces cerevisiae* clearly showed a different contribution of each enzyme to SE hydrolysis under distinct conditions. One difference between the three enzymes is their subcellular localization. While Tgl1p and Yeh1p are LD resident proteins in wild-type cells, the third SE hydrolase Yeh2p is localized to the cell periphery [14]. As there were still some doubts whether Yeh2p is localized rather to the PM or the cER, we re-evaluated the localization of Yeh2p. For this purpose we used a strain deleted in six proteins responsible for the typical formation of the ER association with the PM in wild-type cells. Such a Delta tether strain (*ist2*Δ*scs2*Δ*scs22*Δ*tcb1*Δ*tcb2*Δ*tcb3*Δ) shows a collapsed cER and hence allows to distinguish clearly between PM or cER localization of proteins, especially by fluorescence microscopy. As shown in Figure 2B, the green dotted structures representing a GFP-tagged variant of Yeh2p stain the PM and are still present in this compartment when the cER is condensed in the center of the cell in the Delta tether strain. This finding together with the localization of Yeh2p-GFP to the PM and not to the ER after cell fractionation experiments with the WT tether and Delta tether strains (see Figure 1) confirmed the PM localization of Yeh2p.

Recent studies from our laboratory pointed to a regulation of the two LD localized SE hydrolases Tgl1p and Yeh1p [31] and the TG lipases Tgl3p, Tgl4p and Tgl5p [32,33] by the presence or absence of nonpolar lipids. All these enzymes are retained to the ER in a strain lacking both nonpolar lipids, SE and TG. However, a strain deficient in SE only did not show such a relocalization of Tgl1p and Yeh1p. We wondered, whether Yeh2p might react in a similar way in the QM lacking LD. Figure 5C clearly shows that in cells lacking SE or even both SE and TG, Yeh2p was still present in the PM. One possible explanation for this

localization is that Yeh2p is rather responsible for the hydrolysis of SE coming from outside the cell than for the LD stored SE. Since it is very crucial to supply all different cell compartments with the appropriate amount of sterols in order to keep the membranes properly fluid or rigid, the yeast might be equipped with Yeh2p present at the PM.

One main difference in terms of regulation of the two LD resident SE hydrolases Tgl1p and Yeh1p, and Yeh2p is the expression and protein level. Both, the mRNA levels as well as the protein amounts of Tgl1p and Yeh1p are significantly reduced in the QM strain lacking LD [31]. Interestingly, this effect is not much pronounced in case of Yeh2p. The mRNA level and the protein amount of Yeh2p are only slightly altered in the QM (see Figure 5A, B). This is also true for the strain lacking SE. In the *are1*Δ*are2*Δ strain, the gene expression and protein level of Yeh2p are only slightly reduced compared to wild-type cells.

An interesting observation was made whenever we analyzed Yeh2p by Western blotting. After cell fractionation or after extraction of the proteins from cell homogenate, at least a double band was detectable in Western blots. We used two different tags to be sure that this effect was not specific for one of the used tags. In the case of a GFP-tagged Yeh2p a double band was seen (see Figure 4A), whereas with the very small V5-tag we were even able to detect a triple band, with a prominent upper and lower band framing a very faint band in the middle (see Figure 4B). Not in all cases when a protein is a phosphoprotein a band shift or multiple bands can be seen in Western blots. One indication that phosphorylation plays a role in lipid metabolism is the finding by Kurat *et al.* [49] who tested the phosphorylation status of the TG lipase Tgl4p. These authors demonstrated that Tgl4p is phosphorylated and activated by cyclin-dependent kinase 1 (Cdk1/Cdc28). Their data suggest a general mechanism for coordinating membrane synthesis with cell-cycle progression. We treated Yeh2p samples from wild-type cells with either alkaline phosphatase or lambda protein phosphatase as described in the Materials and Methods section. Due to the disappearance of the upper band and concomitant increase of the signal of the lower one we revealed that Yeh2p is a phosphorylated protein, too. The next steps will be to determine the significance of Yeh2p phosphorylation/de-phosphorylation, and to identify the amino acids modified by phosphorylation as well as the kinase(s) targeting these sites.

Acknowledgments

We thank the Austrian Centre of Industrial Biotechnology (ACIB) Graz for providing the ABI 7500 instrument. The contribution of Helmut Bergler in terms of providing the fluorescence microscopic facility is gratefully acknowledged. This research was funded by the Austrian Science Fund (FWF) projects W901 DK Molecular Enzymology, P23029 to G. D. and P26308 to K. A.

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General Discussion and Summary

The use of the eukaryotic model organism *Saccharomyces cerevisiae* is well acknowledged in the field of basic research. One important pathway that can be studied with the yeast is the lipid metabolic pathway. The ease of achieving deletions in various genes or overexpression strains, the short generation time of the robust yeast and the fact that the principles of lipid metabolism are well conserved between all eukaryotes brought *S. cerevisiae* to the limelight of various challenging questions anticipating their elucidation.

Several researchers contributed to assign the function of the proteins to the respective genes involved in lipid biosynthesis and degradation. In terms of nonpolar lipid synthesis, the four enzymes Are1p, Are2p, Dga1p and Lro1p catalyze the formation of steryl esters (SE) and triacylglycerols (TG), respectively [1]. Dga1p is an acyl-CoA:diacylglycerol acyltransferase acting in an acyl-CoA dependent manner and is dually localized to the ER and to a minor extend to the LD [2,3]. Dga1p is especially active when cells reach the late exponential and stationary growth phase. During the exponential phase, TG are mainly formed by Lro1p [3], a phospholipid:diacylglycerol acyltransferase exclusively localized to the ER [4,5]. Lro1p works in an acyl-CoA independent way and attaches an acyl chain from phosphatidylethanolamine or phosphatidylcholine, respectively, to diacylglycerol [5]. Steryl esters are synthesized in the ER by the two closely related acyl-CoA:sterol acyltransferases Are1p and Are2p [6,7]. Under aerobic conditions, Are2p is the main SE synthase and prefers to esterify ergosterol. In cells deprived of oxygen, mimicked by a heme deficiency mutant, Are1p becomes the more important SE synthase which uses mainly ergosterol precursors as substrate [8–10]. A strain deleted of *ARE1* and *ARE2* completely lacks SE. TG together with SE are then stored in LD, a cell compartment enwrapped by a phospholipid monolayer. Upon requirement, TG and SE can be mobilized by lipolytic enzymes.

Since more than ten years, we know that the three steryl ester hydrolases Tgl1p, Yeh1p and Yeh2p serve to cleave steryl esters [11,12]. However, how the SE synthesizing

enzymes on the one hand and the SE hydrolases on the other hand interact and respond to changes in the SE metabolism was not investigated yet. In the present work we shed light on the regulation of the SE synthases Are1p and Are2p in response to the lack of their counteracting enzymes and, herein, we reported on that in Chapter II. First, we performed lipid analyses in single deletion mutants in SE hydrolases, in a strain lacking SE forming enzymes and in a strain lacking all three SE hydrolases. What we basically expected was to detect no SE in the *are1*Δ*are2*Δ strain, and in case of the triple mutant (TM) *tgl1*Δ*yeh1*Δ*yeh2* lacking the three SE hydrolases a pronounced increase of SE. In fact, the amount of SE was increased in such a strain, however, to our surprise, just slightly up to 130% compared to wild-type levels. Furthermore, we revealed a reduced incorporation of radiolabelled oleic acid and acetate into SE in the TM at the expense of TG. These observations tempted us to speculate that there might be indeed a feedback regulation. Since the regulation can occur at different stages, we started to analyze the gene expression levels of *ARE1* and *ARE2*. However, no marked alterations could be observed. This was also true for the protein levels of Myc-tagged variants of Are1p and Are2p. To go one step further we investigated the *in vitro* enzymatic activity of the two acyltransferases in wild type (WT) and the TM. Not surprisingly, we clearly demonstrated the two acyltransferases to be less active in the mutant devoid of any SE hydrolases. Although both substrates for the acyltransferases, namely sterols and fatty acids, were reduced in the TM, this cannot explain the altered enzymatic *in vitro* activity, because we chose the assay conditions in a way to have the substrates in an excess. What we can provide instead is the evidence of a metabolic and regulatory link between SE metabolism and the biosynthesis of sterols and fatty acids. Figure 1 gives an overview of changes in the pattern of nonpolar lipids and phospholipids (PL) in the strain lacking steryl ester hydrolases. However, we are aware that more efforts are needed to deepen the knowledge about the mechanism of the regulation of the SE metabolism. Despite our experiments, we cannot exclude that a posttranslational modification of one or both of the SE forming enzymes might play a role regarding their reduced activity in the TM. It happens quite often that a phosphorylation alters one protein's activity either positively or negatively. This might also apply to Are1/2p. Cooperative properties of membrane lipids do have the ability to affect the activity of proteins. Hence, investigations of the membrane composition of the ER in the mutant vs the WT could give a valuable hint to shed more light on the mechanistic understanding of ascribed observations. Else, the protein environment implies not only lipids but also other proteins, which might interact in some way with Are1/2p in order to alter their activities.

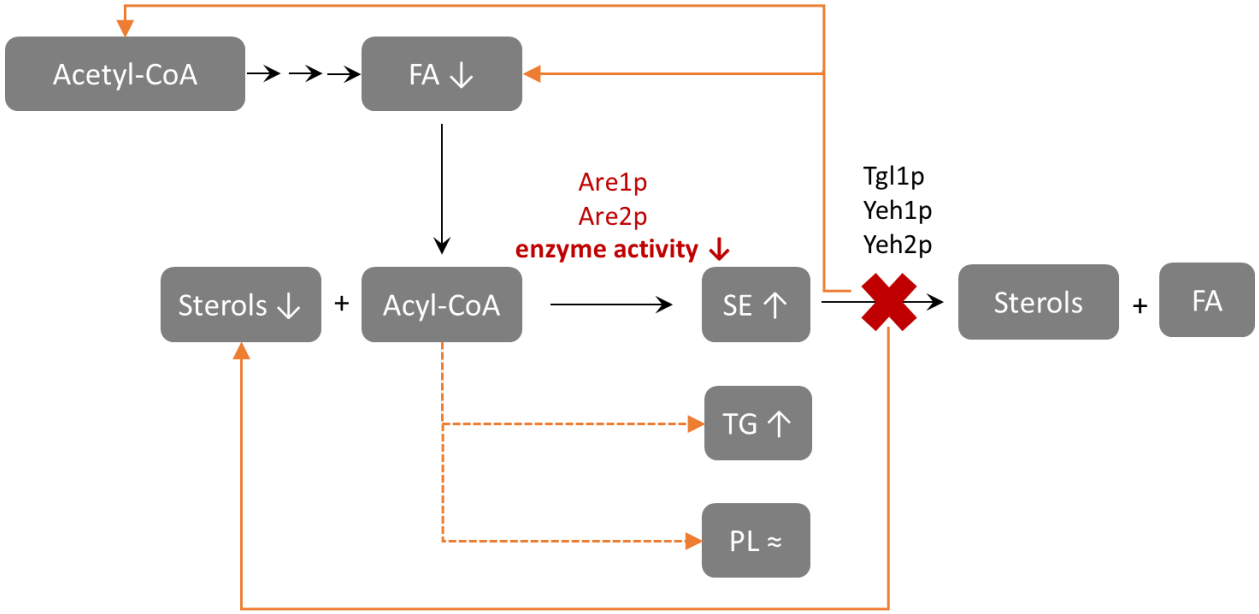

Figure 1 Overview of regulatory effects caused by deletions of *TGL1*, *YEH1* and *YEH2*. Arrows to the top indicate enhanced levels of metabolites (SE, TG) in the TM compared to wild type (WT), arrows to the bottom indicate lowered levels of metabolites (FA, sterols). The amounts of phospholipids (PL) were not markedly altered (indicated by ≈).

Just in the past few years, studies from our laboratory focused on regulatory aspects regarding the lipolytic enzymes responsible for the degradation of TG [13–16]. The lack of LD had several consequences on the main TG lipase Tgl3p. The absence of LD resulted among other effects in a relocalization of Tgl3p from the LD to the ER, where the enzyme lacked lipolytic as well as acyltransferase activity. Hence, the authors proposed that the ER may serve as a parking lot for this enzyme [13]. Tgl4p and Tgl5p experienced the same relocalization to the ER as Tgl3p in a strain devoid of LD and lost their lipolytic activity. However, these two enzymes retained their side activity as lysophospholipid acyltransferase [16]. The relocalization of the three TG lipases is supported by the fact that previous studies had already shown that some typical LD resident proteins are located to the ER in the absence of LD [17,18]. Hence, the close relationship between both before mentioned organelles again became highlighted. As the three steryl ester hydrolases Tgl1p, Yeh1p and Yeh2p still awaited evaluation of their regulation based on the presence or absence of nonpolar lipids, we decided to focus on that aspect throughout one main part of this Thesis. Chapter III describes the fate of Tgl1p and Yeh1p in a strain lacking SE, TG or LD at all. Compared to the three TG lipases, the two LD resident SE hydrolases behaved in a similar way regarding their

relocalization to the ER in a strain lacking LD. To test whether Tgl1p and Yeh1p retained their *in vitro* SE hydrolase activity, we performed assays measuring the cleavage of radiolabelled [1-alpha, 2-alpha (n)-^{3}H]cholesteryl oleate. Since the activity was decreased to the level of our negative control, we concluded that Tgl1p and Yeh1p lost their *in vitro* SE hydrolase activity in the ER in a strain lacking LD. Further, the amount of V5-tagged variants of Tgl1p and Yeh1p were markedly lower compared to WT and the two proteins became highly instable in the quadruple mutant (QM) *are1*Δ*are2*Δ*dga1*Δ*lro1*Δ lacking LD. It has to be pointed out that proteins originally located to LD have to be inserted or attached to a phospholipid monolayer. The ER, however, consists of a phospholipid bilayer. It might be possible that Tgl1p and Yeh1p cannot deal very well with the challenge of a different insertion environment and therefore lose their stability in the ER. However, we found that in the *dga1*Δ*lro1*Δ strain lacking TG, the stability of both SE hydrolases was also markedly reduced, although the proteins were still localized to LD. This raised the speculation that TG are needed to maintain the stability of the proteins. Surprisingly, the SE hydrolase capacity of Tgl1p and Yeh1p was not affected in strains lacking TG, meaning that the absence of TG does not affect the enzymes activity, whereas the relocalization to the ER does. What might be highly interesting to investigate in the future is whether the two LD resident SE hydrolases get somehow posttranslationally modified. Especially for Tgl1p this might be the case as the appearance of a V5-tagged variant of the protein showed a double band in the Western blot for the WT, the strain lacking SE or TG, but not for the strain lacking LD. Either in the strain without LD the amount of protein is that less that the absence of the second band is explainable by the detection limitation of the Western blot, or indeed for example a phosphorylation alters the appearance in the Western blot and the activity of the protein, too. As discussed for the two SE forming enzymes, it is interesting to evaluate whether Tgl1p and/or Yeh1p interact with some other proteins, which might be responsible for the downregulation of the gene expression, the protein amount or activity in the tested strain backgrounds. As summarized in Chapter IV, we also started to analyze the behavior of Yeh2p in the presence/absence of nonpolar lipids. Since there was still some doubt concerning the localization of Yeh2p in WT cells, we again demonstrated that Yeh2p is clearly present in the plasma membrane and not in the cortical ER. Furthermore, we reevaluated the contribution of Yeh2p to *in vivo* SE mobilization. By means of protein phosphatase assays, we managed to postulate that Yeh2p is a phosphorylated protein. Whether the phosphorylation is dependent on cell cycle or growth phase or other conditions, still needs to be investigated. The search for the involved amino acids and the kinase(s) promises an interesting working point for the

future. What we already checked is the localization, the gene expression and the protein amount of Yeh2p tagged variants in strains lacking either SE or LD. There is almost no difference in the respective strain backgrounds, although the gene expression and the protein amount of Yeh2p is slightly reduced in an *are1*Δ*are2*Δ strain and in the QM lacking LD.

In summary, with this Thesis we unraveled novel aspects of the complex metabolic network of nonpolar lipids in the yeast *Saccharomyces cerevisiae* in terms of regulation of the steryl ester metabolism. Since we know and described in the Review article present in Chapter I that a balanced sterol homeostasis is crucial to avoid health complications like Wolman disease, cholesteryl ester storage disease, atherosclerosis, Alzheimer`s disease, Niemann-Pick type C and Tangier disease, we encourage and appreciate any further investigation on sterol homeostasis.

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