Regulation of Steryl Ester Metabolism in the Yeast *Saccharomyces cerevisiae*

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“You never fail until you stop trying.”

Albert Einstein (1879 – 1955)

Danksagung

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Zusammenfassung

Übergewicht, Diabetes, Arteriosklerose und sogar neurodegenerative Erkrankungen sind Folgeerscheinungen eines beeinträchtigten Neutrallipidstoffwechsels. Daher stellen Untersuchungen zum Metabolismus dieser Lipide und zur Regulation der betroffenen Stoffwechselwege eine Herausforderung für Molekularbiologie und Zellbiologie dar. Um diese Probleme zu studieren wurde für die vorliegende Studie die Bäckerhefe *Saccharomyces cerevisiae* als gut akzeptierter und studierter Modellorganismus herangezogen. Die beiden wichtigsten Neutrallipide der Hefe sind Triglyceride und Sterolester. Sie werden von Enzymen des Endoplasmatischen Reticulums synthetisiert und in Lipidpartikeln gespeichert. Triglyceride werden von den beiden Acyltransferasen Dga1p und Lro1p synthetisiert, während Sterolester von den beiden Sterolestersynthase Are1p und Are2p gebildet werden. Beide Neutrallipide, Triglyceride und Sterolester, können bei Bedarf aus Lipidpartikeln mobilisiert werden. Die Triglyceridlipasen Tgl3p, Tgl4p und Tgp5p mobilisieren Triglyceride, und die Sterolesterhydrolasen Yeh1p, Yeh2p und Tgl1p spalten Sterolester. Alle drei Triglyceridlipasen sowie Yeh1p und Tgl1p sind an der Oberfläche von Lipidpartikeln lokalisiert, während Yeh2p eine Komponente der Plasmamembran ist. Das Ziel der vorliegenden Arbeit war das Studium der Regulation des Sterolestermetabolismus. Im Speziellen sollte der Einfluss eines gestörten Abbaus der Sterolester auf deren Synthese untersucht werden. Zu diesem Zweck wurde eine Triplemutante mit Deletionen von *YEH1, YEH2* und *TGL1* verwendet, und der Effekt dieser Deletionen auf die Sterolester bildenden Enzyme Are1p und Are2p untersucht. In der Triplemutante wurde nur ein moderater Anstieg der Sterolester im Vergleich zum Wildtyp verzeichnet. Interessanter Weise konnten auf Transkriptionsebene von *ARE1* und *ARE2* und auf Proteinebene der Genprodukte keine signifikanten Veränderungen in der Triplemutante gezeigt werden. Trotzdem war die enzymatische Aktivität der Acyltransferasen in der Triplemutante stark reduziert. Dieses Resultat deutet auf einen Feedback Regulationsmechanismus auf enzymatischer Ebene hin, wenngleich es den Mechanismus dieser Regulation noch zu klären gilt.

Abstract

Unbalanced levels of non-polar lipids lead to obesity, diabetes, atherosclerosis and even neurodegeneration. Thus, investigations of the complex metabolism of these lipids and the regulation of pathways involved are important issues of modern cell biology and molecular biology. To address these problems in this study, the baker's yeast *Saccharomyces cerevisiae* was used as a well-accepted and intensely investigated model organism. In the yeast, the two major non-polar lipids are triacylglycerols and steryl esters. They are formed by enzymes localized to the endoplasmic reticulum and stored in lipid droplets. Synthesis of triacylglycerols occurs by catalysis of the two acyltransferases Dga1p and Lro1p; and steryl esters are formed by the two steryl ester synthases Are1p and Are2p. Both types of non-polar lipids, triacylglycerol and steryl esters, can be mobilized from lipid droplets upon requirement. The triacylglycerol lipases Tgl3p, Tgl4p and Tgp5p mobilize triacylglycerols, and the steryl ester hydrolases Yeh1p, Yeh2p and Tgl1p cleave steryl esters. All three triacylglycerol lipases as well as Yeh1p and Tgl1p are localized to the surface of lipid droplets, whereas Yeh2p is a component of the plasma membrane. The aim of this Thesis was to shed some light on the regulation of steryl ester metabolism. In particular, we wished to address the question how compromised degradation of steryl esters might affect the synthesis of these lipids. For this purpose, a triple mutant deleted of *YEH1, YEH2* and *TGL1* was used, and the effect of these deletions on the steryl ester forming enzymes Are1p and Are2p was tested. In the triple mutant lacking all three steryl ester hydrolases only a moderately elevated level of steryl esters was detected. Interestingly, neither transcription of *ARE1* and *ARE2* nor protein levels of the respective gene products were affected in the mutant. Nevertheless, the enzymatic activity of the acyltransferases in the triple mutant was strongly reduced. This result indicated a feedback regulation in steryl ester metabolism at the enzyme level although the mechanism remains to be elucidated.

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1. INTRODUCTION

1.1. General overview of lipid classes

Lipids are a group of hydrophobic or amphiphilic small molecules including sterols, waxes, fatty acids, fat-soluble vitamins (such as vitamin A, D, E and K), glycerolipids (monoglycerides, diglycerides and triglycerides), phospholipids (phosphatidic acid, phosphatidylserine, phosphatidylethanolamine, phosphatidylcholine, phosphatidylinositol and cardiolipin as the most prominent ones) and sphingolipids (ceramides, sphingomyelins, glycosphingolipids). Due to the amphiphilic nature of some lipids, they form structures such as vesicles or membranes in an aqueous environment. Lipids also function as storage molecules for energy and can act as signaling molecules.

The budding yeast, *S. cerevisiae*, has become a powerful model system for the elucidation of cell biology, metabolism and regulation of eukaryotic lipids. One considerable advantage of yeast is the availability of gene-enzyme relationships in the pathways for lipid synthesis and turnover. Moreover, regulation and localization of enzymes of lipid metabolism can be studied in the yeast to deepen the knowledge of the role and the fate of lipids. Hence, we decided to make use of the advantages of *S. cerevisiae* throughout this study, with emphasis on non-polar lipids, especially on the regulation of steryl ester metabolism.

1.2. Non-polar lipids in *S. cerevisiae*

Unbalanced levels of non-polar lipids can lead to obesity, diabetes, atherosclerosis and even neurodegeneration. *S. cerevisiae* is a well-accepted model organism to study biochemistry and molecular biology of lipids, especially since there are several homologies between yeast and mammalian cells concerning non-polar lipid metabolism.

Besides steryl esters (SE), triacylglycerols (TG) are the main non-polar lipids in yeast. They are stored in lipid droplets, can be mobilized upon requirement and thus constitute the most important storage lipids in eukaryotic cells.

For TG synthesis, the two precursors phosphatidic acid (PA) and diacylglycerol (DG) are important. PA can be produced via two different pathways. In the glycerol-3-phosphate (G-3-P) pathway, G-3-P from glycolysis is acylated at the *sn*-1 position to lyso-PA (1-acyl-G-3-P) and further converted by a second acylation step in the *sn*-2 position to PA, a key intermediate in lipid metabolism [1]. In the dihydroxyacetone phosphate (DHAP) pathway, DHAP is acylated at the *sn*-1 position. In the next step, acyl-DHAP is reduced by Ayr1p, a 1-acyl-DHAP reductase, to lyso-PA, which can be converted to PA via a further acylation reaction. Pah1p, a phosphatidate phosphatase, forms DG from PA [2,3]. DG can further be acylated by Dga1p [4] and Lro1p [5] to the storage lipids TG. Formation of PA can, however, also be accomplished by hydrolysis of glycerophospholipids by phospholipase D (PLD) [6]. Phospholipase C (PLC) cleaves glycerophospholipids yielding DG [7].

TG are stored within lipid droplets and can be degraded to DG and free fatty acids by three lipases, Tgl3p [8], Tgl4p and Tgl5p [9] (see Figure 1). Recent studies also revealed Ayr1p as another triacylglycerol lipase [10].

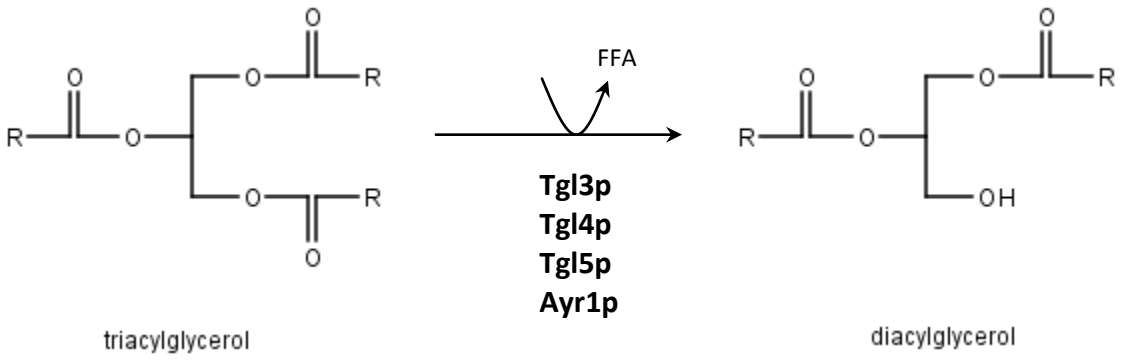

Figure 1. Cleavage of TG into DG and free fatty acid (FFA) by TG lipases Tgl3p, Tgl4p, Tgl5p and Ayr1p. R represents a fatty acid.

Esterification of sterols and hydrolysis of SE, discussed in more detail in chapter *1.5*, are indispensable for cellular sterol homeostasis. On one hand, these processes allow cells to store

chemical energy and membrane components, which can be used in times of deprivation, on the other hand concentrations of free sterols and fatty acids can be balanced. This process is absolutely essential and critical for cell function, as it was shown that elevated levels of free fatty acids [11] and sterols cause lipotoxicity .

1.3. Ergosterol biosynthesis pathway

The most prominent sterol in yeast, predominantly present in the plasma membrane [12], is ergosterol (for structural formula see Figure 2), which is synthesized in a multiple step process involving nearly thirty biochemical reactions. In mammals, the major sterol is cholesterol, and in plants stigmasterol, sitosterol and campesterol are found. Sterols contain a hydroxyl group at the C atom in position 3. The basic structure of steroids is the gonane with its characteristic arrangement of three fused cyclohexane rings and one cyclopentane ring. Common to all sterols is the double bond at C-5,6. The difference between the fungal ergosterol and the mammalian cholesterol is the unsaturation in position C-7,8 in the cyclohexane ring and in position C-22 in the side chain as well as a methyl group at C-24 on the side chain [13].

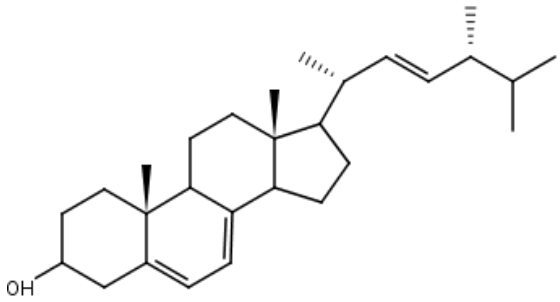

Figure 2. Structural formula of the main fungal sterol ergosterol.

The biosynthesis of ergosterol is mainly accomplished in the ER although some of the needed enzymes are located to lipid droplets. The entire ergosterol biosynthesis pathway can be split in two sections. The pre-squalene pathway containing the mevalonate or isoprenoid pathway converts acetate to farnesyl pyrophosphate. Then, the oxygen dependent ergosterol biosynthesis pathway, commonly termed as post-squalene pathway, further converts farnesyl pyrophosphate through several reactions to ergosterol [2].

The mevalonate pathway (see Figure 3) starts with the conversion of 2 molecules acetyl-CoA to acetoacetyl-CoA mediated by acetoacetyl-CoA thiolase, the gene product of *ERG10*. With another molecule of acetyl-CoA, the intermediate 3-hydroxy-3-methyl-glutaryl-CoA (HMG-CoA) is produced by the enzyme HMG-CoA synthase. HMG-CoA is further converted by HMG-CoA reductase, an enzyme which is subject to feedback inhibition by ergosterol [14], yielding mevalonic acid. Also in mammalian cells HMG-CoA reductase is a well-studied enzyme which can be inhibited by agents called statins [15]. A double phosphorylation followed by a decarboxylation reaction is needed to form isopentenyl pyrophosphate (IPP), which gets isomerized to dimethylallyl-pyrophosphate. The *ERG20* gene product is responsible for conversion of 2 molecules of IPP, first forming geranyl pyrophosphate and secondly farnesyl pyrophosphate, the starting molecule of the second section of the ergosterol biosynthesis pathway.

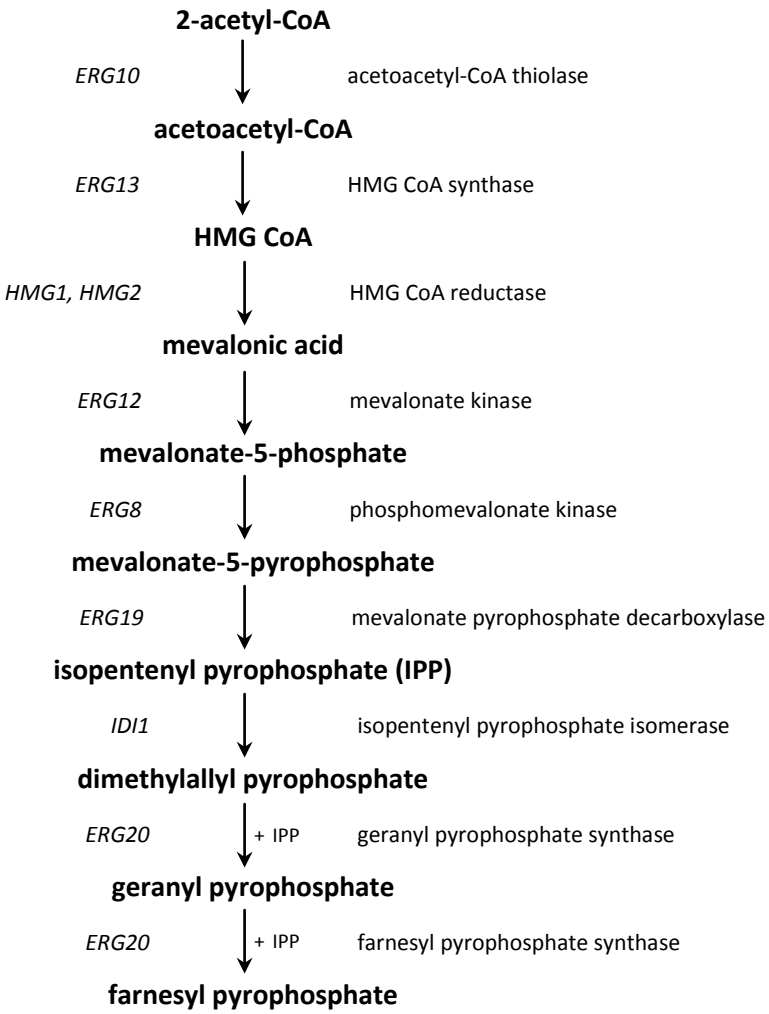

Figure 3. The pre-squalene pathway from acetyl-CoA to farnesyl pyrophosphate (modified after [2]).

ERG9 encodes squalene synthase which converts farnesyl pyrophosphate to squalene through cleavage of pyrophosphate. Subsequently, squalene epoxide is synthesized by the enzyme squalene epoxidase, which can be inhibited by allylamines such as terbinafine [16,17]. Squalene epoxide gets then converted into lanosterol, which is reduced after a demethylation step. After a reaction catalyzed by three enzymes (methyloxidase, dehydrogenase and ketoreductase) the ergosterol precursor zymosterol is synthesized. Sterol C-24 methyltransferase encoded by *ERG6* yields fecosterol, which is isomerized to episterol. Followed by two desaturations and one reducing reaction, the end product ergosterol is synthesized.

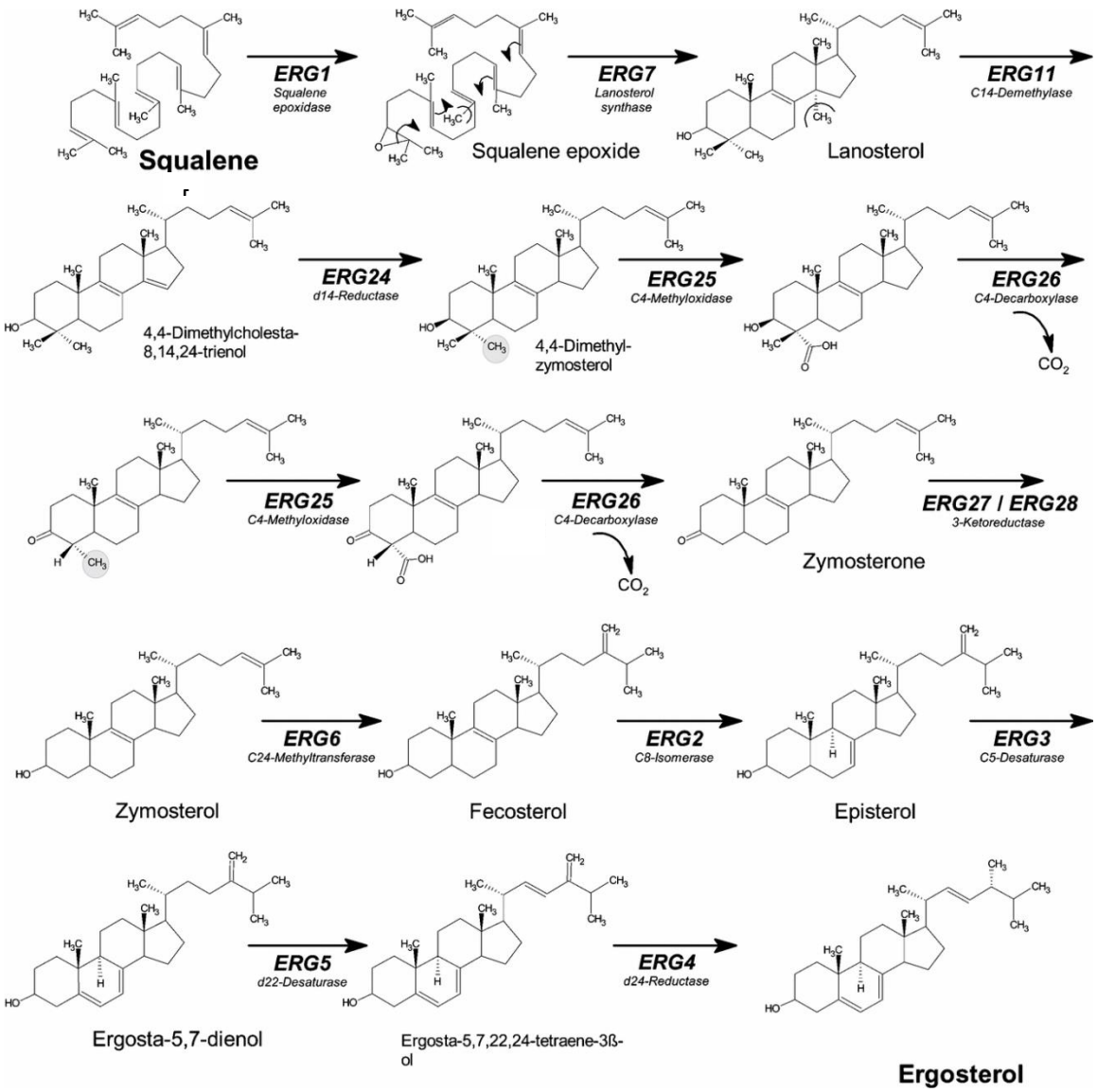

Figure 4. The post-squalene pathway [18].

1.4. Fatty acid biosynthesis

Fatty acids are indispensable components for conserving energy. They form the hydrophobic part in membrane bilayers and also serve as signaling molecules [19]. *S. cerevisiae* mainly harbors C_{16} and C_{18} fatty acids, whereof about 80% are monounsaturated due to the action of the Δ9 desaturase Ole1p [20]. Fatty acids can either be set free by lipolysis, taken off from external sources, or synthesized *de novo*. The *de novo* synthesis, as shown in Figure 5, is accomplished mainly in the cytosol, whereas elongation is localized to the ER. Enzymes involved in fatty acid synthesis are components of a complex called fatty acid synthase (FAS) composed of Fas1 (β subunit) and Fas2 (α subunit) organized in a hexameric α6β6 complex.

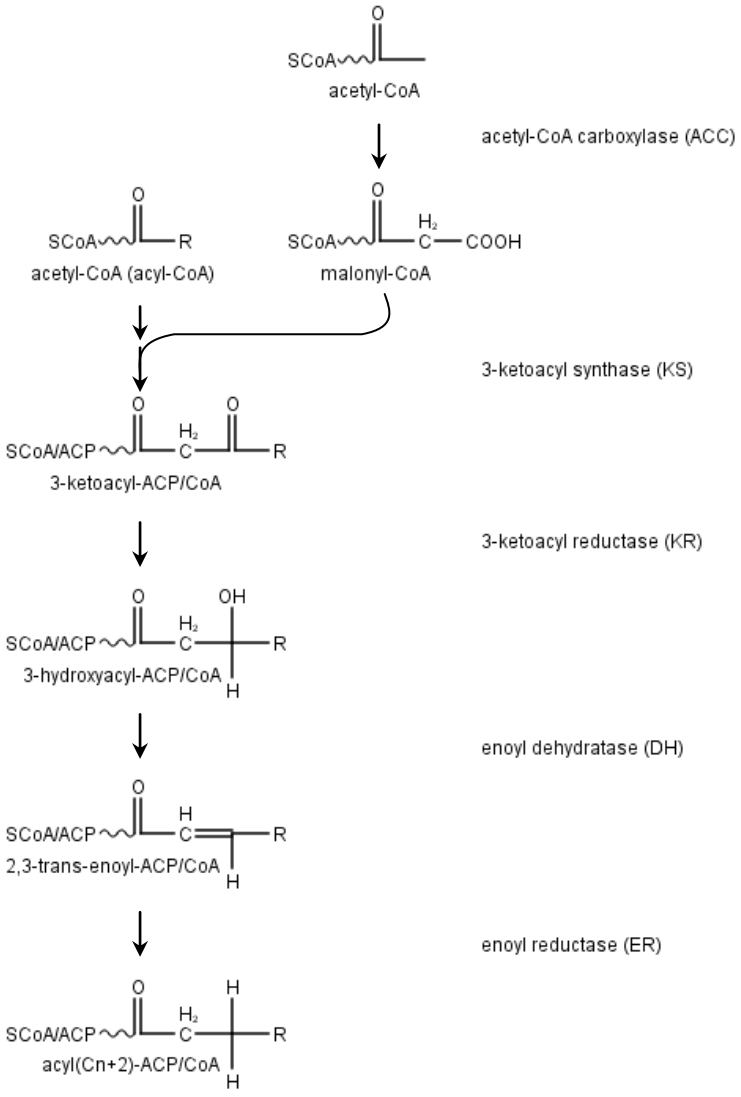

Figure 5. Reaction scheme of fatty acid synthesis and elongation.

In the initial step, acetyl-CoA is carboxylated to malonyl-CoA, which is further converted to 3-ketoacyl-CoA catalyzed by 3-ketoacyl synthase. Through the reduction of the 3-ketoacyl-CoA to 3-hydroxyacyl-CoA, dehydratation to enoyl-CoA, and a second reduction, a saturated acyl-chain extended by two carbon atoms is built.

1.5. Enzymes related to steryl ester metabolism

According to the Nomenclature Committee of the International Union of Biochemistry and Molecular Biology (NC-IUBMB), enzymes are divided in six groups depending on the reaction they catalyze [1] (Table 1). Two of these families, EC 2 and EC 3, are important for steryl ester metabolism.

Table 1. Enzyme classification by the Nomenclature Committee of the International Union of Biochemistry and Molecular Biology (NC-IUBMB).

Enzyme Commission Number	*Enzyme family*
EC 1	Oxidoreductases
EC 2	Transferases
EC 3	Hydrolases
EC 4	Lyases
EC 5	Isomerases
EC 6	Ligases

Figure 6 gives an overview of enzymes involved in steryl ester metabolism in *S. cerevisiae.*

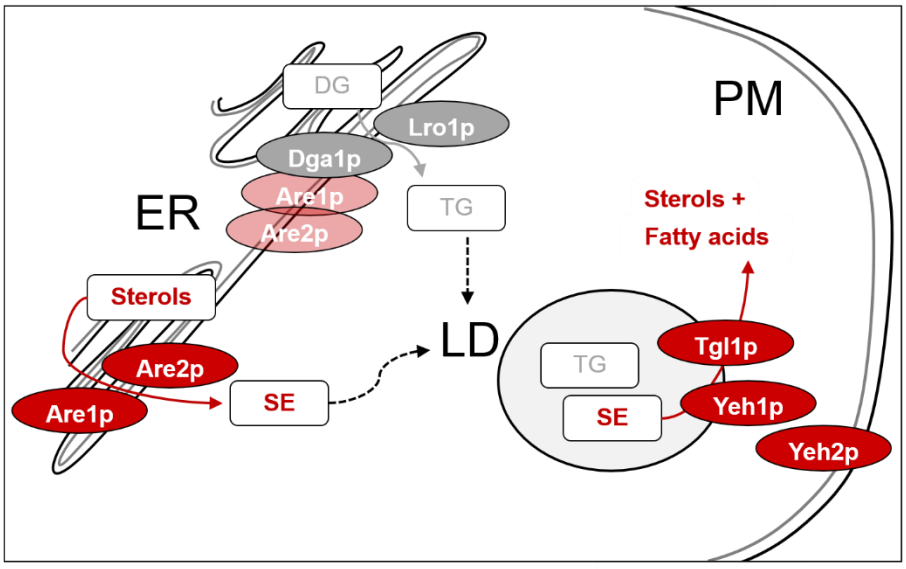

Figure 6. Overview of enzymes related to steryl ester metabolism.

The two ER-associated acyl-CoA:sterol acyltransferases Are1p and Are2p are responsible for the synthesis of steryl esters from sterols and activated fatty acids. The three steryl ester hydrolases Tgl1p, Yeh1p and Yeh2 carry out the cleavage of steryl esters yielding sterols and free fatty acids.

1.5.1. The two acyltransferases Are1p, Are2p

In yeast, the two ER located acyl-coenzyme A:cholesterol acyltransferase (ACAT)-related enzymes Are1p and Are2p are responsible for the formation of steryl esters in an acyl-CoA dependent reaction by adding an activated free fatty acid to the 3-OH group of sterols [21,22] (see Figure 7).

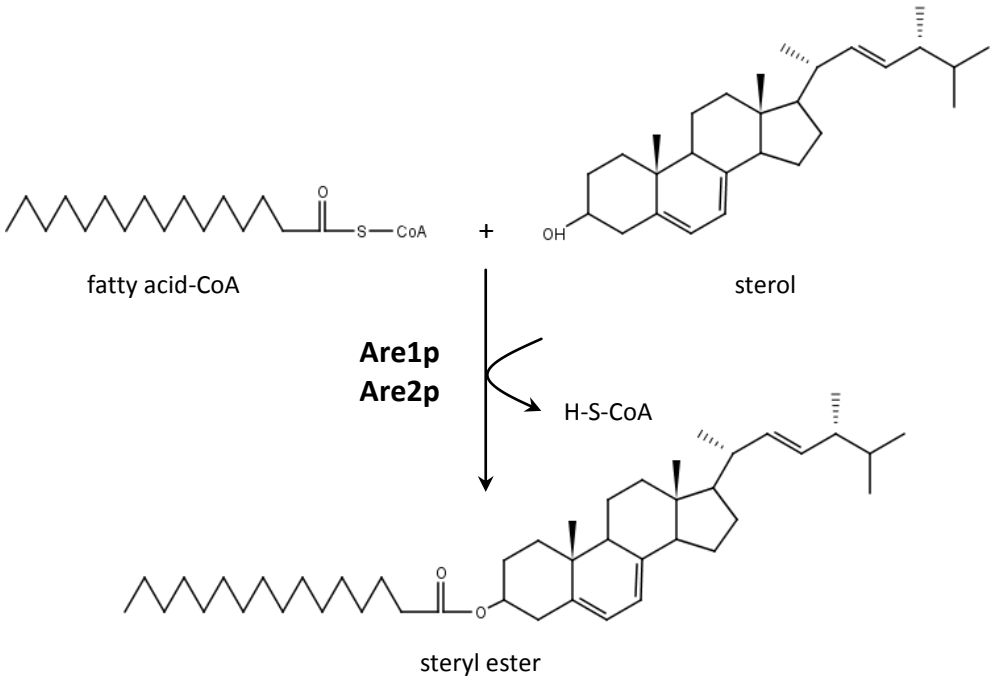

Figure 7. Formation of steryl esters by Are1p, Are2p in yeast.

The two *ARE*-genes show 49% sequence identity, and *ARE1* and *ARE2* exhibit high homology of approximately 20% to the human ACAT. Are2p is the major catalytic isoform, accounting for at least 65-75% of total cellular activity under standard growth conditions [23,24]. In *S. cerevisiae*, accumulation of steryl esters occurs in the stationary phase and during sporulation [25]. Yeast steryl esters accumulate in lipid droplets [26], an organelle that is structurally related to human

lipoproteins. A transcriptional regulation of *ARE* genes by heme is known. While the *ARE1* transcription is increased in heme-deficient and anaerobic cells, the *ARE2* gene transcript is more abundant in aerobic cells competent for heme synthesis [27–29]. Are2p has a significant preference to esterify ergosterol, whereas Are1p shows substrate specificity to sterol precursors, mainly lanosterol, but also uses ergosterol and other precursors as substrate [22]. A *Δare1Δare2* strain does not show any detectable amounts of steryl esters but is still viable [21,22]. It was demonstrated that lack of sterol esterification in yeast leads to a decrease of total cellular sterol due to a 3-fold down regulation of *ERG3* expression in a *Δare1 Δare2* strain [30].

1.5.2. The three steryl ester hydrolases Tgl1p, Yeh1p and Yeh2p

The three steryl ester hydrolases Tgl1p, Yeh1p and Yeh2p, carry out the biochemical conversion of steryl esters to sterols and free fatty acids. All three hydrolases belong to the alpha/beta hydrolase superfamily, which contains eight beta sheets connected by helical loops. The three amino acids histidine, aspartic acid and serine form a typical catalytic triade in the active site of the enzymes.

The highest steryl ester hydrolase activity was detected in the plasma membrane and attributed to Yeh2p [12,31]. Yeh1p and Tgl1p are located to LD and are paralogues of the mammalian acid lipase family, with the lysosomal acid lipase and the gastric lipase as two prominent representatives. Yeh1p constitutes the main steryl ester hydrolase under heme depletion [32]. Tgl1p has also TG lipase activity, although the steryl ester hydrolase activity is predominant [24].

The three different yeast SE hydrolases show specificities for substrates. Yeh2p exhibits a slight preference to cleave zymosteryl esters [22] similar to Tgl1p, although the latter enzyme also uses ergosteryl esters and other esters of sterol precursors. Zymosteryl and ergosteryl esters are also utilized by Yeh1p, but this enzyme cleaves fecosteryl esters more efficiently than Yeh2p and Tgl1p. Lanosteryl esters are not a preferred substrate of any of the three hydrolases [33].

However, under anaerobic conditions, Yeh1p is equally active against lanosteryl and ergosteryl esters [34].

1.6. Lipid droplets – the organelle of lipid storage

Lipid droplets, also termed lipid particles or oil bodies, are the storage organelle for the two main non-polar lipids in yeast, TG and SE. TG, which form the core of LD, are surrounded by ordered shells of steryl esters. LD are covered by a phospholipid monolayer. The hydrophobic core is composed of nearly equal amounts of SE and TG. The average size of LD ranges from 300 – 400 nm [26,35]. A quadruple mutant *Δdga1Δlro1Δare1Δare2* lacking all four genes responsible for non-polar lipid anabolism, is devoid of LD indicating that the formation of LD is closely related to non-polar lipid synthesis [36].

Lipid droplets contain a variety of proteins associated with or embedded in the surface phospholipid monolayer. LDs can be isolated to high purity allowing the determination of associated proteins under different experimental conditions. Such proteomic studies indicated that proteins of LD are responsible for interactions of LDs with other organelles, and also propose a function of LDs as storage organelles for proteins, or as traps for unfolded or aggregated proteins [37]. LD-localized proteins do not contain conserved targeting sequences. Hydrophobic sequences that harbor targeting information are thought to act as control of targeting. Deletion of such sequences abolishes the localization of the truncated proteins to the LD [38–42]. However, homologues to mammalian perilipins were not found [3].

As a link to non-polar lipid metabolism, the three TG lipases encoded by the genes *TGL3, TGL4,* and *TGL5* as well as the SE hydrolases Yeh1p and Tgl1p were found to be localized to lipid droplets [43,9,44,32]. These facts indicate that LD are not only an inert storage structure for non-polar lipids, but also a metabolically active organelle [45].

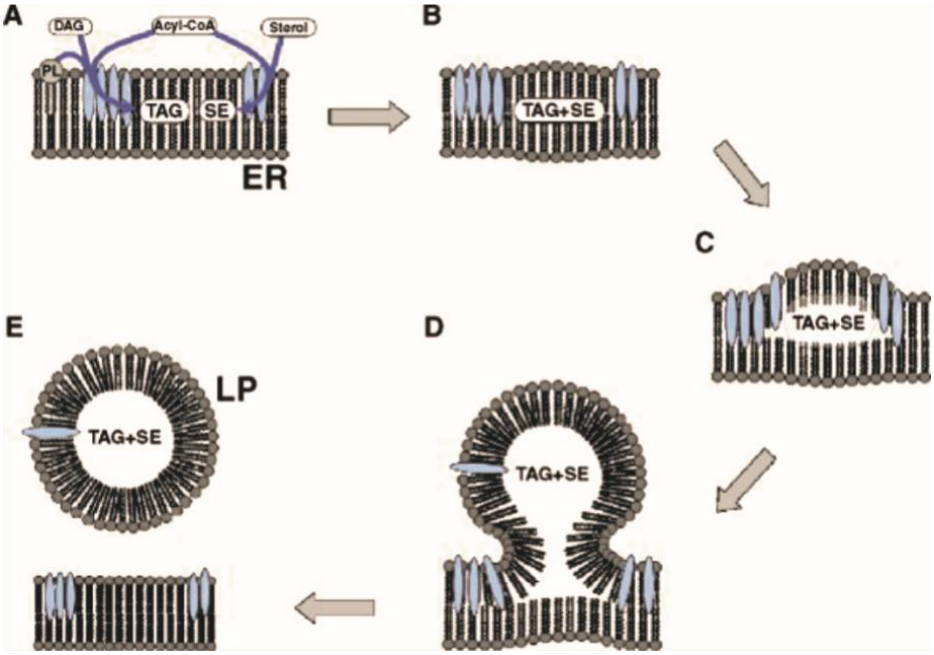

Figure 8. Model of lipid droplet biogenesis [46].

Several hypothesis of lipid droplet biogenesis exist based on recent research. However, the exact mechanism is still unclear. Many lines of evidence support the view that LD are derived from the ER [40,47–50]. A possible model is shown in Figure 8. Since TG and SE are synthesized in the ER, they are supposed to be enriched between the two leaflets of the ER membrane. Reaching a critical amount of non-polar lipids, lipid droplets emerge which then pinch off the ER and are released into the cytoplasm. The LD surface monolayer is derived solely from the cytosolic leaflet of the ER membrane, at least in this model [46]. The so called “bicelle” model suggests that LD are excised from the ER, leaving behind a gap in the membrane, which immediately can be filled up [51].

2. OBJECTIVES

In recent years, several studies about non-polar lipids and the enzymes involved in their metabolism were performed. Consequently, pathways leading to the formation of these lipid classes were elucidated, and most of the enzymes involved were identified and characterized. Nevertheless, there are still many open questions especially concerning the regulation of non-polar lipid metabolism. Hence, the aim of this Master Thesis was to shed light on some of these aspects with a focus on the metabolism of SE and its regulation.

This study is based on previous investigations of our laboratory which identified the major enzyme players in non-polar lipid metabolism of the yeast *Saccharomyces cerevisiae*. The major question addressed in this Thesis was how the two SE synthesizing enzymes of the yeast, Are1p and Are2p, behave when SE degradation is blocked. A strategy was developed to use different mutant strains lacking one, two or all three SE hydrolases (Yeh1p, Yeh2p and Tgl1p) and strains containing tagged variants of the two SE synthesizing acyltransferases. With these biological tools we planned to perform lipid analyses, gene expression studies, protein quantification by Western Blot analyses as well as enzyme activity assays.

In the course of this Thesis we wanted to get an overview of the lipid profile in the triple mutant (TM) lacking all three steryl ester hydrolases and in single deletion strains lacking either Yeh1p, Yeh2p or Tgl1p. As we proposed a broader link between non-polar lipid and phospholipid metabolism, triacylglycerols and phospholipids were also quantified in wild type and triple mutant. These experiments were supplemented by *in vitro* labelling assay aimed at quantification of lipid formation rates in strains lacking SE hydrolysis. Altogether, this study should lead us to a better understanding of non-polar lipid metabolism in the yeast, which might also have a broader impact on the lipid metabolism in other cell types.

3. MATERIALS

Materials used throughout this Master Thesis are listed in Table 2 – Table 8, Figure 9 and Figure 10.

3.1. Equipment

Table 2. Devices used in this work.

Device	*Description and company*
Magnetic stirrer	MR3001 K, HeidoLDh
Vortex	Minishaker IKA MS1, Merck
Centrifuges	Table centrifuge Fresco 17 Thermo scientific; Heraeus
	Rotina 46R, Hettrich Zentrifugen
	Function line laborfuge 400R, Heraeus
	RC6 Plus, Sorvall (Rotor F21S, F10S)
Autoclave	MLV 5075, Systec
Thermo mixer	Thermo mixer comfort 1,5 ml, Eppendorf
Thermostat	Thermostat 5320, Eppendorf
Microwave	Panasonic
Pipettes	Pipetman 1000 µl, 200 µl, 20 µl, 10 µl, Gilson
Shaker	VXR basic, IKA, VIBRAX
	Multitron standard, Infors HT
Photometer	Spectrometer U-1100, Hitachi
	Specord 210
pH meter	SA520, Orion
Oven	Kelvitron, Heraeus
Scale	GP3202, Sartorius
	Toledo AG135, Mettler
PCR device	Thermo cycler 2720, Applied biosystems
	Primus 25 advanced, Peqlab
	ABI 7500, Applied Biosystems for qRT-PCR
Scanner	Scanjet 7400c, HP
	TLC-Scanner, CAMAG TLC-Scanner 3
Camera	Data-recorder DR-2, Shimadzu
	GelDoc 2000 BioRad
SDS-PAGE	BioRad Mini Protean II equipment
Scintillation counter	LSC Safety, Baker, Deventer, The Netherlands

3.2. Chemicals and Kits

Table 3. Chemicals used in this work.

Company	*Chemical*
Carl Roth GmbH + Co. KG, Karlsruhe	Acetic acid, acetone, acrylamide (Rotiphorese 40, 37.5:1), agar-agar, agarose, ampicillin, APS, boric acid, bromphenol blue, CHAPS, diethyl ether, DTT, EDTA, Roti Safe GelStain, glucose, glycerin, HCl, KCl, KH_2PO_4, K_2HPO_4, L-arginin, L-histidine, lithiumacetate, L-leucin, mercaptoethanol, MES, methanol, milk powder, $MnCl_2$, NaCl, NaOH, PEG, petroleum, plastic cuvette, Ponceau S, SDS, sulfuric acid, TCA, Tris
Merck KGaA, Darmstadt	TLC Silica gel 60 plates
Oxoid Deutschland GmbH, Wien	Peptone, yeast extract
Mast Group Ltd., Bootle, UK	Tryptone
Sigma-Aldrich Handels GmbH, Wien	Triton X-100, Oleoyl-CoA
THP Medical Products, Wien	Yeast nitrogen base
Fresenius Kabi Austria GmbH, Graz	Aqua Bidest.
AustrAlco Österreichische Agrar-Alkohol HandelsgesmbH, Spillern	Absolute ethanol
Amresco Inc., Cochran, USA	Sorbitol
Pall Austria Filter GmbH	BioTrace NT nitrocellulose blotting membrane
Whatman GmbH, Dassel	Filter paper
Greiner bio-one	Eppendorf tubes, PS tubes 15 mL and 50 mL
Thermo Scientific	SuperSignal® West Pico Chemiluminescent Substrate
Seikaguku Corporation	Zymolyase 20T
Perkin Elmer Life Science	[^{14}C]oleoyl-CoA

Table 4. Kits used in this work.

Company	*Kit*
MACHEREY-NAGEL GmbH & Co. KG, Düren, Germany	NucleoSpin Gel and PCR Clean-up
Invitrogen	SuperScript® III Platinum® SYBR Green One-Step qRT-PCR Kit
Quiagen	Quiagen-Micro Kit RNeasy

3.3. Strains

Table 5. Strains used in this work.

Strain	*Genotype*	*Source*
Wild type	BY4741 Mat a; *his3Δ1; leu2Δ0; met15Δ0; ura3Δ0*	Euroscarf
TM	BY4741; *tgl1Δ::kanMX4; yeh1Δ::kanMX4; yeh2Δ::kanMX4*	Wagner *et al* .[33]
Δare1Δare2	BY4741; *are1Δ::kanMX4; are2Δ::kanMX4*	Wagner *et al.* [33]
Δtgl1	BY4741; *tgl1Δ::kanMX4*	Euroscarf
Δyeh1	BY4741; *yeh1Δ::kanMX4*	Euroscarf
Δyeh2	BY4741; *yeh2Δ::kanMX4*	Euroscarf
WT Are1p-Myc	BY4741; *ARE1-13Myc::HIS3MX6*	This study
WT Are2p-Myc	BY4741; *ARE2-13Myc::HIS3MX6*	This study
TM Are1p-Myc	TM; *ARE1-13Myc::HIS3MX6*	This study
TM Are2p-Myc	TM; *ARE2-13Myc::HIS3MX6*	This study

3.4. Culture media

Table 6. Culture media.

YPD medium	
Medium composition	1% (w/v) yeast extract, 2% (w/v) peptone, 2% (w/v) glucose
Plates	YPD-medium, 2% (w/v) agar
SD medium	
Medium composition	2% glucose, 0.67% yeast nitrogen base, respective amino acid supplements
Plates	SD-medium, 2% (w/v) agar

3.5. Antibodies

Table 7. Antibodies used in this work.

Primary antibodies
Mouse anti-Myc Antibody (Fa. Roche) 1:1000
Rabbit anti-Wbp1 Antibody (Fa. Roche) 1:1000
Secondary antibodies
Goat anti-mouse Antibody 1:5000
Goat anti-rabbit Antibody 1:5000

3.6. Primers

Table 8. Primers used in this work.

Quantitative RT-PCR		
Primer	*Sequence (5' → 3')*	t_m
Are1_RTFw	CTTCACTGTTCTGTACATGCTCACGTTTTACATG	60.5°C
Are1_RTRev	CGAAACGCAATTCCACCAGTCGCCGTAG	64.7°C
Are2_RTFw	GATGTATCCTGTAGCAATGAGAGCATTGGCTGTG	62.9°C
Are2_RTRev	CTGGGACGATATCAACGAGCAATCCAAC	60.9°C
ACT1_RTFw*	CCAGCCTTCTACGTTTCCATCCAAG	59.5°C
ACT1_RTRev*	GACGTGAGTAACACCATCACCGGA	60.5°C
Amplification from pFA6a-13Myc-HIS3MX6		
Primer	*Sequence (5' → 3')*	t_m
are1fw*	TTGGTGTCTGTTCAGGGCCCAGTATCATTATGACGTTGTACCTGACCTTACGGA	69.4°C
are1rev*	TTGTATATCTATCAAGGGCTTGCGAGGGACACACGTGGTATGGTGGCAGTATCG	69.5°C
are2fw*	TCGGTATCTGCATGGGACCAAGTGTCATGTGTACGTTGTACTTGACATTCCGGA	69.6°C
are2rev*	AAAATTTACTATAAAGATTTAATAGCTCCACAGAACAGTTGCACGATGCCATCG	64.7°C
Colony PCR for checking positive clones		
Primer	*Sequence (5' → 3')*	t_m
are1fw_con*	GACCGCAGTTGTCCAACG	56.8°C
are2fw_con*	CAGAACCATAATCGGAAATGTTAT	51.2°C
13-Myc_rev*	TATTTAGAAGTGGCGCGAATTCAC	55.6°C

* Special thanks to Claudia Schmidt for providing primers.

3.7. Standards

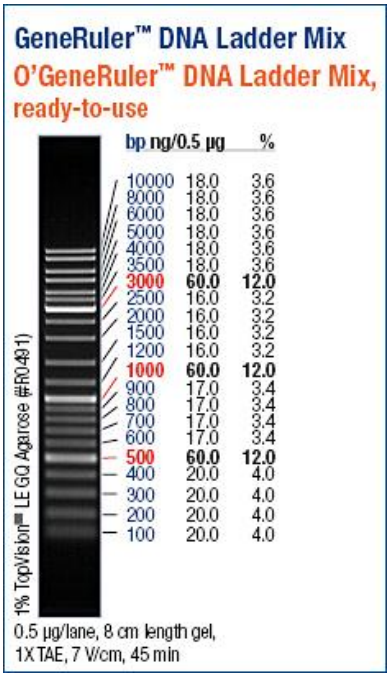

Figure 10. GeneRuler DNA Ladder Mix (Fermentas)

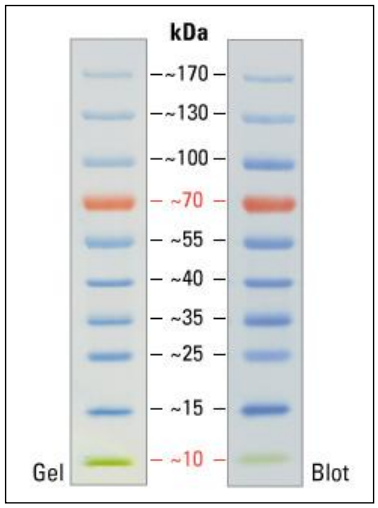

Figure 9. „PageRuler Prestained Protein Ladder“ (Fermentas)

4. METHODS

4.1. Genetic techniques

4.1.1. PCR and Primer design

Polymerase chain reaction is one of the most important molecular biology tools for amplifying DNA. Through several cycles of heating for denaturing the DNA and cooling for primer annealing and DNA synthesis the amount of DNA can be exponentially increased.

4.1.1.1. Quantitative Real time PCR

To investigate the gene expression level, RNA was isolated from a cell suspension grown to the exponential growth phase (for detailed steps see *4.1.2*). To compare the amount of RNA from different strains, RNA is reversely transcribed to DNA whereof a distinct fragment is amplified by using SuperScript® III Platinum® SYBR® Green One-Step qRT-PCR Kit[1]. Therefore, specific oligonucleotides providing the 3' hydroxyl group for the start of the DNA-polymerase reaction and the appropriate nucleotides are necessary. For real time PCR these primers should fulfill some special properties. The optimal melting temperature is 66°C. At the beginning and the end of the primer a cytosine or a guanine is preferable. The amplified product including the primers should have a size of about 100 bp and be located in the last third of the gene. Primers for amplifying parts of the sequence of *ARE1* and *ARE2* in the TM are listed in *3.6*. It is noteworthy that in our study the melting temperature was calculated by Oligo Analyser. However, the melting temperature is slightly higher if calculated by Eurofins MWG Operon, where primers were ordered. To ensure the optimal melting temperature of 66°C, we designed our primers with a melting temperature slightly under 66°C. PCR components are shown in Table 9.

[1] http://tools.lifetechnologies.com/content/sfs/manuals/superscript_onestep_sybr_man.pdf

Table 9. PCR components and used volumes for quantitative Real time PCR.

Volume	*PCR components*
0.5 µL	SuperScript® III RT/Platinum® Taq Mix
12.5 µL	2x SYBR Green Reaction Mix
0.05 µL	ROX Reference Dye (25 µM)
5 µL	RNA template (6 ng/µL)
5.95 µL	RNase free water (DEPC treated)
0.5 µL	Forward Primer (10 µM)
0.5 µL	Reverse Primer (10 µM)
25 µL	**Total Volume**

Reactions were performed in sealed MicroAmp Optical96-well Reaction Plates and measurement was accomplished using an ABI7500 instrument. For appropriate heating and cooling the following PCR program was used:

cDNA synthesis	50°C	3 min	
Initial denaturation step	95°C	5 min	
Denaturation	95°C	15 sec	} 40 x
Primer annealing and elongation	60°C	30 sec	
Final extension	40°C	1 min	

Quantification was done using the $\Delta\Delta c_t$ method as described by Livak and Schmittgen [52].

4.1.1.2. Amplification of the 13-Myc tag

For chromosomal tagging of Are1p and Are2p the PCR-mediated method described by Longtine *et al.* [53] was used. For amplification of the 13-Myc tag from the Longtine plasmid, a forward primer consisting of the last few nucleotides of the gene of interest and a homologues part to the beginning of the 13Myc-His3MX6 section in the plasmid was designed. Reverse primers are homologue to the downstream region of the ORF from the gene of interest and to the last few nucleotides from 13Myc-His3MX6 section. In this case, besides all the other crucial components for doing a PCR, ExTag polymerase with proof reading activity was used for DNA synthesis (see Table 10).

Table 10. PCR components and used volumes for amplification of the 13-Myc tag.

Volume	*PCR components*
1 µL	pFA6a-13Myc-His3MX6 (10 ng/µL)
5 µL	10x Buffer
2.5 µL	dNTPs (2.5 mM)
2.5 µL	Forward Primer (10 µM)
2.5 µL	Reverse Primer (10 µM)
33.5 µL	dH_2O
0.5 µL	ExTag polymerase
50 µL	**Total Volume**

The following PCR program served for amplifying the target sequence:

Initial denaturation step	98°C	30 sec	
Denaturation	99°C	10 sec	30 x
Primer annealing	55°C	30 sec	
DNA synthesis	72°C	2 min	
Final extension	72°C	10 min	
Cool down	4°C	∞	

4.1.1.3. Colony PCR

To check whether the amplified 13-Myc tag was integrated into the chromosomal genome of the yeast strains at the appropriate position, colony PCR was carried out. To identify positive clones after homologues recombination tiny amounts of colonies from agar plates (SD minus histidine) not older than 24 hours were picked and incubated for 15 min at 37°C in 50 µL zymolyase suspension (2.5 mg/mL H_2O) using PCR tubes. After a 1 min centrifugation step at 5,000 rpm the supernatant was removed and samples were incubated for 5 min at 92°C. The pellet served as DNA template for PCR with components listed in Table 11. A primer binding to the target gene served as forward primer. The reverse primer annealed to a sequence within the open reading frame of the 13-Myc tag.

Table 11. PCR components and used volumes for colony PCR.

Volume	*PCR components*
25 µL	Maxima Hot Start PCR Mix
2.5 µL	Forward Primer (10 µM)
2.5 µL	Reverse Primer (10 µM)
19 µL	dH_2O
49 µL	**Total Volume**

Initial denaturation step:	95°C	4 min	
Denaturation:	95°C	30 sec	30 x
Primer annealing:	55°C	30 sec	
DNA synthesis	72°C	1 min	
Final extension:	72°C	10 min	
Cool down:	4°C	∞	

4.1.2. RNA isolation

The desired yeast strains were pre-cultured in approximately 10 mL of SD medium with all amino acids supplemented for 16 h at 30°C. To make cells ready for RNA isolation, 25 mL of SD medium with all amino acids supplemented were inoculated with the appropriate volume of the preculture to an OD_{600} of 0.2 and incubated at 30°C. This main culture was grown to an OD_{600} between 3 and 6 to reach the exponential growth phase. Cells from the 25 mL main culture were harvested in a sterile 50 mL Sarstedt tube at 4,500 rpm for 5 min. After discarding the supernatant with the water-jet pump, pellets were washed with about 30 mL of sterile water. Cells were again harvested by centrifugation, and the resulting cell pellet was frozen. RNA isolation was performed with the RNA Isolation Quiagen-Micro Kit RNeasy[2]. To avoid contaminations and degradations of RNA it is important to work on ice and use only RNase free water and tubes. Solutions used are mentioned in Table 12. The frozen pellet was thawed on ice and transferred 2 times with 200 µL RLT-buffer, each, to an Eppendorf tube with cooled glass beads. Cells were broken for 10 min with the disrupter genie at 4°C. After a short step of centrifugation, supernatants were transferred into a new cooled Eppendorf tube. Cell debris were sedimented by a centrifugation step of 2 min at top speed. The supernatant was again pipetted into a new Eppendorf tube to mix 1:1 with 70% EtOH. This suspension was applied to a RNeasy MinElute spin column placed in a 2 mL collection tube and centrifuged for 15 sec at top speed. After discarding the flow through, 350 µL of RW1-buffer was added to the RNeasy MinElute spin column followed again by a centrifugation step at top speed for 15 sec. DNase digestion was carried out by pipetting a gently mixed solution of 10 µL DNase (10 µg/µL) with 70 µL RDD-buffer to the column. DNase digestion was performed at 30°C for 30 min. Subsequently, 350 µL RW1-buffer were added and centrifuged at top speed for another 15 sec. The flow through was discarded. 500 µL RPE-buffer were added to the column and again

[2] http://www.qiagen.com/products/catalog/sample-technologies/rna-sample-technologies/total-rna/rneasy-micro-kit#resources

centrifuged at top speed for 15 sec. After adding 500 µL of 80% EtOH, a 2 min centrifugation step at top speed followed. A new collection tube was used to collect the flow through of a second 10 min lasting centrifugation step at top speed. Columns were put into an Eppendorf tube, and after 3 min of incubation RNA was eluted with 20 µL of RNase free water by centrifugation for 1 min at top speed. The concentration of RNA was determined and RNA was frozen at -70°C.

Table 12. Buffers used for RNA isolation.

Buffers	*Components*
RLT-Buffer	10 µL ß-Mercaptoethanol, 990 µL RLT-Buffer (RNeasy Mini Kit, Qiagen)
70% EtOH	700 µL EtOH abs., 260 µL RNase free H_2O
80% EtOH	960 µL EtOH abs., 240 µL RNase free H_2O

4.1.3. Yeast transformation

For yeast transformation the high-efficiency lithium acetate transformation protocol [54] was employed. The yeast strains were pre-cultured in approximately 10 mL of YPD medium for 16 h at 30°C. A main culture containing 50 mL YPD medium was inoculated to an OD_{600} of 0.1. After incubation at 30°C, cells were harvested at an OD_{600} between 0.6 and 0.8 by centrifugation at 4,500 rpm for 5 min at RT. The pellet was washed with 20 mL of sterile water, centrifuged as mentioned before, and the water was discarded. The pellet was suspended in 1 mL of 0.1 M LiAc solution (0.1 M LiAc in 10 mM Tris/HCl, pH 7.5 plus 1 mM EDTA) and transferred into a 1.5 mL Eppendorf tube. After harvesting the cells at top speed for 30 sec, the pellet was again suspended in 1 mL of 0.1 M LiAc solution and centrifuged. Finally, the pellet was suspended in 400 µL 0.1 M LiAc solution. The transformation mix (Table 13) was added to 50 µL of the above prepared cell suspension. After regeneration for 30 min at 30°C, cells were heat-shocked for 20 min at 42°C. The cells were collected by centrifugation at 3,000 rpm for 15 sec. Subsequently, the transformation mix was discarded and the pellet was suspended in 300 µL sterile water. 100 µL of this suspension was plated onto selective media (SD minus histidine) and incubated at 30°C for two to three days. Single colonies were tested for correct recombination by colony PCR.

Table 13. Components and volumes of the transformation mix.

Volume	*Components*
240 µL	PEG (50% w/v in H_20)
36 µL	1 M LiAc solution (1 M LiAc in 10 mM Tris/HCl, pH 7.5 plus 1 mM EDTA)
10 µL	Salmon DNA (10 mg/mL) as carrier DNA (denatured for 10 min at 95°C and kept on ice before using)
400 – 700 ng	Purified PCR product

4.1.4. Agarose gel electrophoresis

Agarose gel electrophoresis is a convenient molecular biology method to separate nucleic acid fragments of different sizes and estimate their size by using a standard of fragments with known size (see Figure 10). The concentration of cross-linked agarose polymers determines the pore size of the casted gel. An electric field is used to pull the negatively charged nucleic acids through the gel matrix. The smaller a fragment, the faster it migrates through the gel allowing a separation by size. For our purpose, gels of 1% agarose prepared as listed in Table 14 were used. Instead of the toxic and cancerogenic ethidium bromide, Roti®-Safe GelStain[3] was used to bind in the minor groove of helical nucleic acids to visualize the DNA fragments. DNA samples for amplification of the 13-Myc tag (amplified with ExTag Polymerase) were mixed with 6x loading dye from Fermentas to a final concentration of 1x loading dye. The Maxima Hot Start PCR Mix still contains a loading dye. For analytical and preparative gels the amperage was set to 70 mA and 90 mA, respectively, for about 30 to 45 min.

Table 14. Buffers for agarose gel electrophoresis.

Buffer	*Components*
10x TBE	108 g Tris, 55 g boric acid, 40 mL 0.5 M EDTA (pH 8.0)
1% Agarose	1 g agarose, 100 mL 1x TBE

[3] http://www.carlroth.com/website/de-ch/pdf/Roti_SafeGelStain_E.pdf

4.1.5. Purification of DNA from agarose gels

Amplified fragments from Longtine plasmid pFA6a-13Myc-His3MX6 containing the Myc tag for chromosomal tagging were cut out of a 1% preparative gel. Therefore, the gel was examined under low energy PrepUVlight avoiding mutations in the DNA. Bands of the desired fragment were cut out with a scalpel. For purification the NucleoSpin Gel and PCR Clean-up[4] Kit was used following the manufacturer's instructions.

4.2. Biochemical methods

4.2.1. TCA protein precipitation and protein quantification by the method of Lowry

Proteins were quantified by the method of Lowry *et al.* [55]. Homogenate samples were diluted 1:10, whereas samples from microsomal fractions were diluted 1:5. Water was added to the appropriate volume of samples up to 400 µL. Proteins were precipitated by adding 100 µL of ice-cold 50% (w/v) trichloroacetic acid. Incubation was done either over night or at least for 1 hour on ice. To isolate the proteins, a 10 min centrifugation step at 13,000 rpm at 4°C followed. The supernatant was evaporated and 100 µL of solution C (0.1% SDS in 0.1 M NaOH) were added and incubated for 30 min, or at least until the pellet was dissolved, at 37°C. In test tubes 300 µL water, 100 µL of the protein suspension, and 2 mL of solution A (Table 15) were mixed by vigorous vortexing. After 10 min incubation at RT, 200 µL solution B (Folin-Ciocalteu-phenol reagent, 1:1 diluted) were added and samples were again mixed. Photometrical measurement at 546 nm was done after a 30 min incubation step at RT, and the protein amount was calculated from a calibration curve generated with BSA.

Table 15. Solutions for TCA protein precipitation and protein quantification by the method of Lowry.

Solution	*Components*
TCA	50% (w/v) trichloroacetic acid
Solution A (10 samples)	200 µL disodiumtartrat (2.2%), 200 µL $CuSO_2 \cdot 5H_2O$ (1%), 500 µL SDS (20%), 20 mL Na_2CO_3 (2%) in 0.1 M NaOH
Solution B	Folin-Ciocalteu-phenol reagent, 1:1 diluted
Solution C	0.1% SDS in 0.1 M NaOH

[4] http://www.mn-net.com/Portals/8/attachments/Redakteure_Bio/Protocols/DNA%20clean-up/UM_PCRcleanup_Gelex_NSGeLDCR.pdf

4.2.2. SDS-PAGE and Western blot analysis

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) is a convenient method to separate biological macromolecules, especially proteins, by electrophoretic mobility. Stacking gels (5%) and separating gels (12.5%) were prepared as described in Table 16. Electrophoresis was performed as described by Laemmli [56] using an amperage of about 40 mA for one gel.

Table 16. Components of SDS-gels.

Stacking gel	*5%*	*Separating gel*	*10%*	*12.5%*
dH_2O	3.690 mL	dH_2O	3.610 mL	2.990 mL
Tris/HCl pH 6.8	625 µL	Tris/HCl pH 8.8	3.750 mL	3.750 mL
Acrylamide/Bis	562.5 µL	Acrylamide/Bis	2.500 mL	3.120 mL
20% SDS	25 µL	20% SDS	50 µL	50 µL
10% APS	25 µL	10% APS	48 µL	48 µL
TEMED	10 µL	TEMED	10 µL	10 µL

Proteins of microsomal fractions were precipitated with TCA as mentioned in section 4.2.1. Again, TCA precipitation was employed over night or for at least for 1 hour. Subsequently, a 10 min centrifugation step at 10,000 rpm at 4°C was performed. The pellet was washed with 1 mL ice-cold sterile water to remove the acid. For final preparation of the samples for SDS-PAGE, the pellet was suspended in an appropriate volume of sample buffer (Table 17). Prior to loading onto the gel, samples were incubated at 37°C for 30 min. After SDS-PAGE proteins were blotted onto a nitrocellulose membrane as described by Haid and Suissa [57]. For this purpose a transfer cassette was built as follows:

1. Transfer cassette with the black site on the bottom
2. Sponge
3. Whatman filter paper
4. SDS gel
5. Membrane
6. Whatman filter paper
7. Sponge
8. Closing of the cassette

To check successful blotting and to estimate whether protein samples of equal amounts were loaded on the gels, the membrane was stained with Ponceau S. Prior to blocking the membrane either over night at 4°C or at least for 1 hour with blocking solution, the Ponceau S dye was washed away with dH_2O. After blocking, the membrane was washed three times with the washing solution for 5 min. Mouse anti-Myc antibody (Fa. Roche) 1:1000 diluted was used as primary antibody to bind the Myc antigen. Rabbit anti-Wbp1 antibody (Fa. Roche) 1:1000 diluted recognized the Wbp1p antigen. The membrane was incubated with the primary antibodies for 1 hour with shaking. Then, the membrane was again washed three times with the washing solution for 5 min. Goat anti-mouse antibody and goat anti-rabbit antibody, both diluted 1:5000 and conjugated with the enzyme peroxidase, were used to bind the appropriate primary antibody. After antibody incubation for 1 hour, the membrane was again washed three times with the washing solution for 5 min. SuperSignal® West Pico Chemiluminescent Substrate was added onto the membrane and incubated for approximately 3 min. The chemiluminescence was detected using an X-ray film. After developing, washing and fixing the X-ray film, black bands at the appropriate size were visible.

Since Wbp1 (WW domain binding protein 1) is an abundant protein only in the ER, it provides an ER specific marker protein. It was used to determine whether equal amounts of proteins were loaded onto the gel.

Table 17. Solutions used for SDS-PAGE and Western blot.

Solutions	*Components*
Sample buffer	780 µL dissociation buffer, 200 µL Tris/HCl, pH 8.8, 20 µL ß-mercaptoethanol
5x running buffer, 1 L	15 g Tris, 1.87 g EDTA, 71 g glycine, 2.5 g SDS
Dissociation buffer	20 mM KH_2PO_4 (pH 6.8), 6 mM EDTA, 6% SDS, 10% glycerol, 0.05% bromphenol blue
10x Stock solution, 1 L	140 g glycerol, 30 g Tris, in dH_2O
Transfer buffer	100 mL 10x stock solution, 200 mL methanol, 0.5 mL 20% SDS solution, 700 mL dH_2O
10x TBS	1.5 M NaCl, 0.5 M Tris, pH 7.5
TBST	1x TBS, 0.1% (v/v) Triton X-100
Blocking solution	1x TBS, 5% (w/v) milk powder
Washing solution	TBST
Ponceau S	0.1% (w/v) Ponceau S, 5% acetic acid, in dH_2O
Detection solutions	SuperSignal® West Pico Chemiluminescent Substrate

4.2.3. Lipid extraction from total cell extracts

Lipids were extracted from total yeast cell extracts following the method of Folch *et al.* [58]. The desired yeast strains were pre-cultured in approximately 10 mL of SD medium with all amino acids supplemented for 16 h at 30°C. 25 mL of SD medium with all amino acids supplemented were inoculated with the appropriate volume of the preculture to an OD_{600} of 0.1 and incubated at 30°C for 26 hours to the stationary growth phase. Cells from 25 OD units of the cell culture were harvested by centrifugation at 4,500 rpm for 5 min at RT. To disintegrate the cells, the pellet was suspended with 400 µL dH_2O. The same amount of glass beads was added and put for 10 min on the disrupter genie at 4°C. The whole content from the Eppendorf tubes was transferred to Pyrex glass-tubes. 4 mL chloroform:methanol (2:1, v/v) were used as extracting agent, and samples were vortexed for 1 hour at RT with the Vibrax to extract lipids to the organic phase. Prior to a 5 min centrifugation step at 1,500 rpm, 1.5 mL 0.034% magnesium chloride solution were added. The upper aqueous phase was removed by aspiration followed by a washing step with 2 mL of artificial upper phase (Table 18) for 10 min at the Vibrax to remove the proteins. After centrifugation for 5 min at 1,500 rpm, the upper phase was discarded again and the organic phase was transferred to a new Pyrex glass-tube. To get as much lipids as possible extracted, the same extracting procedure was repeated for a second time with only 2 mL chloroform:methanol (2:1, v/v) for only 10 min. Finally, lipid

extracts were dried under a stream of nitrogen and either stored at -20°C or used for non-polar lipid analysis.

Table 18. Solutions used for lipid extraction of total cell extracts.

Solution	*Components*
Chloroform:Methanol	$CHCl_3$/MeOH (2:1, v/v)
Magnesium chloride solution	0.034% $MgCl_2$
Artificial upper phase	$CHCl_3$/MeOH/H_2O (3:48:47, v/v/v)

4.2.4. Non-polar lipid analysis

After lipid extraction, the dried lipid samples obtained from cells according to 25 OD_{600} units, were dissolved in an appropriate volume of $CHCl_3$/MeOH (2:1, v/v). Solved lipids were stored in GC-vials to eventually repeat non-polar lipid analysis. Lipid extracts of 4 OD_{600} units cell culture were spotted onto Silica gel 60 TLC plates. Chromatograms were developed in an ascending manner by using solvent A (see Table 19) for half of the distance. Then, plates were dried with a hair-dryer and developed to the top of the plate using solvent B as a solvent system. After plates were dried again, they were dipped for 15 s into the charring solution. After incubating the plates in a heating chamber at 105°C for at least 30 min, bands were quantified densitometrically by scanning at 400 nm with a TLC Scanner. Cholesteryl oleate served as standard for steryl esters.

Table 19. Solvents for neutral lipid analysis.

Solvent	*Components*
Solvent A	Light petroleum:diethyl ether:acetic acid/35:15:1 (v/v)
Solvent B	Light petroleum:diethyl ether/49:1 (v/v)
Charring solution	0.63 g $MnCl_2 \cdot 4\ H_2O$, 60 mL water, 60 mL methanol, 4 mL conc. sulfuric acid

4.2.5. Quantification of lipid phosphorus

To estimate the total amount of lipid phosphorus, analyses were performed according to Broekhuyse [59]. Strains were pre-cultured in approximately 10 mL of SD medium with all amino acids supplemented for 16 h at 30°C. 200 mL of SD medium with all amino acids

supplemented were inoculated with the appropriate volume of the preculture to an OD_{600} of 0.1. The main culture was incubated for 26 h at 30°C to the stationary phase. Cells were harvested by centrifugation at 4,500 rpm for 5 min. The cell wall was disintegrated by using 5 mL breaking buffer (Table 20) and 5 µL PMSF as a protease inhibitor. Cells were disintegrated at 4°C at the disrupter genie in the presence of glass beads. The supernatant was collected in a 15 mL Sarstedt tube, centrifuged at 4,500 rpm for 5 min. The supernatant of this centrifugation step served as cell-free homogenate for further experimental steps. For lipid extraction as described above, 0.8 mg of protein were used. However, only solvent A (light petroleum/diethyl ether/acetic acid (35:15:1, per vol.)) was used to the top of the Silica 60 plate to develop the chromatogram. After sprinkling the plate with phosphate free dH_2O, bands according to phospholipids were scraped off and transferred into phosphate free glass tubes. The samples were dried for approximately 15 min at 105°C. Next, 0.2 mL of acid mixture (90 vol% conc. H_2SO_4, 10 vol% 72% $HClO_4$, Table 20) were added. Lipids were charred for 30 min at 180°C in the heating block moving the tubes every 10 min. In case that the acid mixture had a light yellow color, one to three drops of H_2O_2 were used as oxidizing agent. After cooling down to RT, 4.8 mL of a molybdate/ANSA solution was added, properly vortexed and incubated for 30 min at 105°C. Silica gel was sedimented by centrifugation at 1,200 rpm for 5 min. The absorption of the supernatant was measured at 830 nm. Inorganic phosphate served as a standard (73 mg $K_2HPO_4 \cdot 3H_2O$ solved in 10 mL aqua dest., 1 µg/µL). Since the correlation between phosphate and phospholipids had not yet been considered, the amount of µg phosphate per mg protein had to be multiplied with the factor 25 to yield the amount of phospholipid/mg protein.

Table 20. Chemicals used for phosphate determination.

Solution	*Components*
Breaking buffer	50 mM Tris/HCl, 150 mM NaCl, pH 7.4
Acid mixture (H_2SO_4:$HClO_4$)	90 vol% conc. H_2SO_4, 10 vol% 72% $HClO_4$
Molybdate solution	0.26% ammonium heptamolybdate $(NH_4)_6Mo_7O_{24} \cdot 4H_2O$
ANSA solution	40 g/250 mL potassium metabisulfite $K_2S_2O_5$, 0.63 g/250 mL ANS (Anilinonaphthalene sulfonic)-acid, 1.25 g/250 mL sodium sulfite Na_2SO_3
Molybdate/ANSA solution	500 parts of molybdate solution + 22 parts of ANSA solution
Phosphate standard	73 mg $K_2HPO_4 \cdot 3H_2O$ solved in 10 mL aqua dest. Concentration of the stock solution: 1 mg/mL

4.2.6. Cell fractionation – preparation of microsomes

Cell fractionation by differential centrifugation is a powerful method for organelle isolation. Since our proteins of interest are located to the ER, preparation of microsomes was performed following published procedures [60]. Therefore, pre-cultures were grown in 25 mL YPD for 24 h at 30°C. Main cultures with 4 – 6 times 500 mL YPD were inoculated with 5 mL pre-culture and incubated for 22 h at 30°C to the late exponential growth phase. Cells were harvested by centrifugation at 5,000 rpm for 5 min with the SLC-3000 rotor, washed with dH_2O followed again by a centrifugation step. Cell wet weight (cww) was determined. Cells were suspended with twice the volume of pre-warmed buffer A (Table 21). 1.54 mg DTT/mL buffer A were used. The suspension was incubated for 10 min at 30°C with shaking. After centrifugation at 5,000 rpm for 5 min, the pellet was suspended and washed with buffer B, again followed by a centrifugation step. To digest the cell wall, the cell pellet was suspended in an appropriate volume buffer B (cww divided by 0.15) containing at least 2 mg Zymolyase 20T/g cww and incubated for 1 h at 30°C with shaking. When using microsomes to further perform Western Blot analysis, PMSF (1 M, 2 µL/g cww) was added when using buffer C to avoid protein degradation by proteases. For harvesting the spheroplasts, suspension was centrifuged at 4°C with 5,000 rpm for 5 min and washed with ice-cold buffer B. Spheroplasts were homogenized on ice in a Dounce homogenizer with the same volume of buffer C as cww with a loose fitting pestle with 15 strokes. To remove cell debris, unbroken cells and nuclei the homogenate was centrifuged at 5,000 rpm for 5 min at 4°C. The resulting pellet was suspended twice in buffer C, homogenized and centrifuged again. Combined supernatants were centrifuged at 13,000 rpm for 30 min in an SS34 rotor to remove the mitochondrial fraction. The supernatant was centrifuged at 16,000 rpm for 30 min yielding a small microsomal pellet (30,000 g fraction), which was suspended in an appropriate volume of Tris/HCl, pH 7.4. Aliquots of microsomal fractions and homogenate were shock frozen in liquid nitrogen and stored at -70°C.

Table 21. Buffers used for microsomal preparation.

Buffers	*Components*
Buffer A	0.1 M Tris, pH 9.4 with H_2SO_4
Buffer B	1.2 M Sorbitol, 20 mM KH_2PO_4, pH 7.4 with KOH
Buffer C	0.6 M Mannitol, 10 mM Tris, pH 7.4 with HCl
Tris/HCl	10 mM Tris, pH 7.4 with HCl

4.2.7. Acyl-CoA:ergosterol acyltransferase Assay

The measurement of acyl-CoA ergosterol acyltransferase relies on the incorporation of radioactivity from [^{14}C]oleoyl-CoA into steryl esters. Ergosterol served as the second substrate.

Table 22. Ergosterol mix.

Volume	*Ergosterol mix components*
50.0 µL	2.5 mM Ergosterol (solved in $CHCl_3$/MeOH 2:1) -> dried under a stream of nitrogen
487.5 µL	0.1 M KPO_4 buffer, pH 7.4
12.5 µL	200 mM CHAPS solution

An ergosterol mix (Table 22) was prepared. 40 µL of the ergosterol mix was added to 108 µL 0.1 M KPO_4 buffer (pH 7.4), 2 µL [^{14}C]oleoyl-CoA, 10 µL oleoyl-CoA and 40 µL DTT (15 mg/mL) as reducing agent (Table 23). To start the assay, either 70 µg protein of microsomal fraction or 250 µg protein of homogenate suspended in a total volume of 200 µL were mixed properly with the substrate and incubated for 15 min at 30°C. The reaction was stopped by adding 3 mL $CHCl_3$/MeOH (2:1, v/v), and lipids were extracted for 1 h by shaking on the Vibrax every 10 min. After adding 1 mL of 0.034% $MgCl_2$ solution, samples were centrifuged for 5 min at 1,300 rpm. Subsequently, the aqueous upper phase was removed by aspiration and the organic phase containing the lipids was dried under a stream of nitrogen. 40 µL of $CHCl_3$/MeOH (2:1, v/v) were used to suspend the dried lipids. They were spotted onto a Silica gel 60 plate, and the chromatogram was developed by using light petroleum/diethyl ether/acetic acid (35:15:1, per vol.) as solvent system. After reversible staining with iodine vapor, bands according to steryl esters were scraped off the plate and suspended in 8 mL scintillation cocktail containing 5% water. Finally, samples were subjected to LSC Safety scintillation counting to measure the amount of incorporated [^{14}C]oleoyl-CoA to steryl esters as a measure for the activity of the acyltransferases.

Table 23. Substrate components and used volumes for one assay.

Volume	*Substrate components*
40 µL	Ergosterol mix
40 µL	DTT (15 mg/mL)
108 µL	0.1 M KPO_4 buffer, pH 7.4
2 µL	[^{14}C]oleoyl-CoA (0.02 µCi/µL)
10 µL	Oleoyl-CoA (2 mg/mL)
200 µL	**Total substrate volume for one reaction**

5. RESULTS

5.1. Lipid analyses in strains bearing defects in steryl ester metabolism

5.1.1. Quantification of steryl esters in strains deficient in steryl ester hydrolysis

First of all, we wanted to determine and quantify the amount of steryl esters in strains impaired in steryl ester hydrolysis. *S. cerevisiae* BY4741 acted as the reference and was termed wild type (WT). The *Δare1Δare2* strain was used to show that this strain lacks the ability to synthesize steryl ester. For steryl ester analysis, lipids were extracted from total cell extracts as described above. Extracted lipids were separated by thin layer chromatography. Figure 11 is representative of one Silica Gel 60 plate after charring the organic compounds. Cholesteryl oleate served as standard. To measure the density of the bands in a range of linear correlation, different amounts of the standard solved in $CHCl_3$/MeOH (2:1, v/v) were applied to the Silica Gel 60 plate, and a calibration line was calculated.

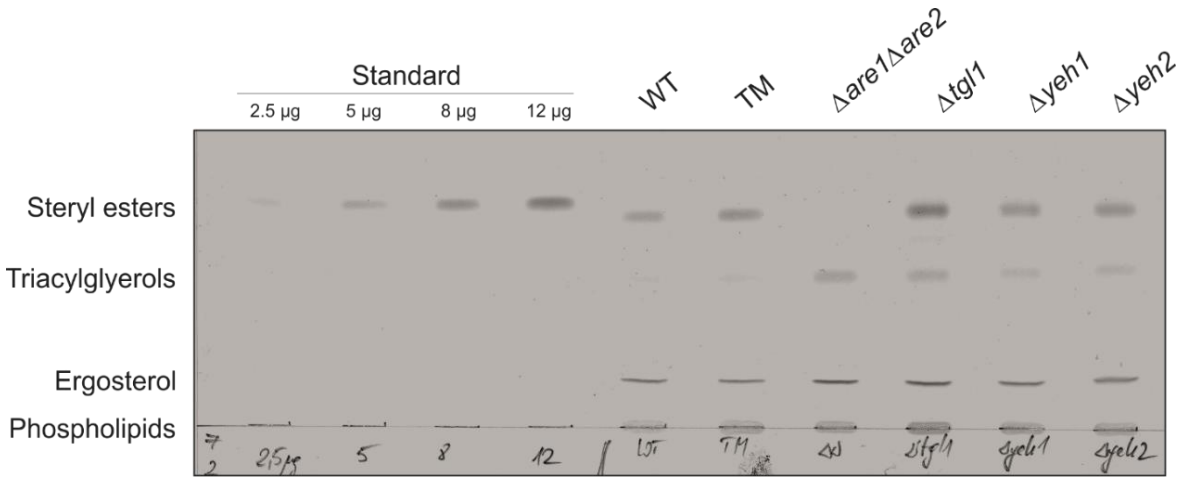

Figure 11. Non-polar lipid analysis by thin layer chromatography showing one Silica Gel 60 plate. Cells were grown in SD medium with all amino acids supplemented to the stationary growth phase. Lipids were extracted by the method of Folch *et al.* [58]. Extracted lipids of 4 OD_{600} units cell culture of were spotted onto the Silica Gel 60 plate. Cholesteryl oleate served as standard.

Amounts of SE from WT were set to 100%. As shown in Figure 12, a *Δare1Δare2* strain lacking both genes responsible for SE formation, did not produce any SE. The TM lacking all three known SE hydrolases showed a slight increase in SE to about 120%. A *Δtgl1* single mutant strain

seemed to behave like the TM having elevated amounts of SE. A *Δyeh1* and *Δyeh2* strain, however, appeared like WT with hardly unaffected amounts of SE. Data obtained in this study showed the same tendency as results from previous studies in our laboratory, although those changes were more pronounced. The differences to results obtained by Wagner *et al.* [33] could be due to other cultivation media. In their studies YPD or MMGal (minimal media with 2% galactose) was used, whereas in our studies SD medium with all amino acids supplemented was chosen as growth medium.

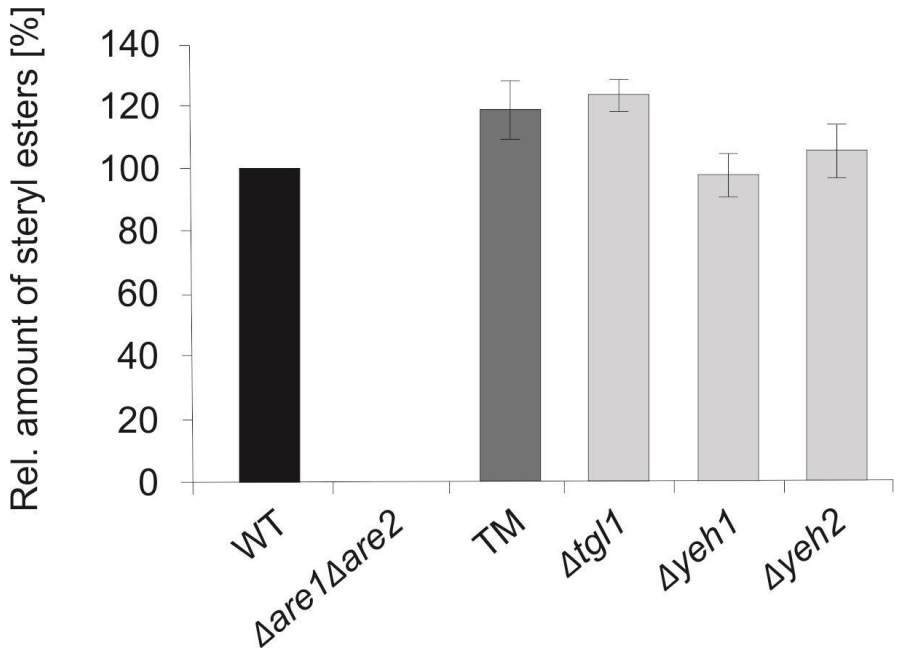

Figure 12. Steryl ester analysis by TLC. The relative amount of SE in WT (black bar) was set at 100%. The amount of SE compared to WT was measured in a strain deficient of the two acyl-CoA:sterol acyltransferases *Δare1Δare2* (0%), in a strain lacking all three steryl ester hydrolases (*Δtgl1Δyeh1Δyeh2* dark grey bar 116%) and in strains lacking one of the steryl ester hydrolases each (*Δtgl1* 124%, *Δyeh1* 97%, *Δyeh2* 104%). Data shown are mean values of three independent experiments performed in duplicates with the respective deviation as indicated.

5.1.2. Quantification of lipid phosphorus

To get an idea how non-polar lipid and phospholipid metabolism are connected, the amounts of phospholipids in the TM and in a *Δare1Δare2* strain were determined and compared to WT. Figure 13 shows that the TM deficient in all three SE hydrolases did not affect the total phospholipid level. In contrast, the *Δare1Δare2* strain had a higher level of total lipid

phosphorus. The increase to about 130% can be explained by the fact that more free fatty acids are available in this strain and utilized to build phospholipids and TG instead of SE.

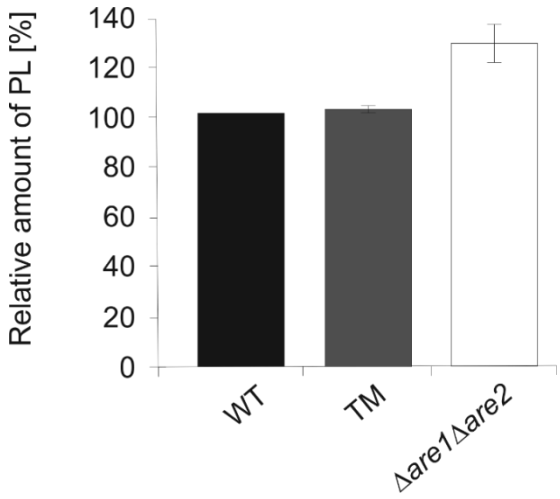

Figure 13. Total lipid phosphorus determination of homogenates from WT (black bar 100%), TM (dark grey bar 100%, D 2%) and *Δare1Δare2* (white bar 126%, D 7%). 0.8 µg of protein were used. Lipids were extracted by the method of Folch *et al.* [58]. Chromatograms were developed to the top in an ascending manner using the solvent system light petroleum/diethyl ether/acetic acid (35:15:1; per vol.). Phospholipid spots were scraped off and analyzed by the method of Broekhuyse [59]. Data shown are mean values of two independent experiments performed in duplicates with the respective deviation as indicated.

5.2. Expression of *ARE1* and *ARE2* in the steryl ester hydrolase mutant strain

For the analysis of relative gene expression levels, we decided to make use of the qRT-PCR mediated technique using the SuperScript® III Platinum® SYBR® Green One-Step qRT-PCR Kit from Invitrogen. The great advantage of this system is that both cDNA synthesis and PCR are performed in a single tube. It is not absolutely necessary to have the mRNA purified since SuperScript® III Reverse Transcriptase is not significantly inhibited by rRNA or tRNA. Platinum® Taq DNA polymerase is responsible for amplifying the DNA, and SYBR® Green served as fluorescent dye that binds directly to double-stranded DNA. The signal generated by the dye is proportional to the DNA concentration during PCR. One of the most critical steps is to get the whole DNA digested during RNA isolation to avoid wrong calculation of the RNA concentration to be used for the qRT-PCR and to minimize unspecific binding of the primers.

Analysis was performed as described in section *4.1.1.1.* Calculation using the $\Delta\Delta c_t$ method revealed results as shown in Figure 14. Panel A shows the comparison of the expression level of *ARE1* in TM and WT; and in panel B the expression level of *ARE2* is shown. Since the

transcription of none of the two acyltransferases was significantly altered, we excluded the influence of the triple mutation on the transcription level.

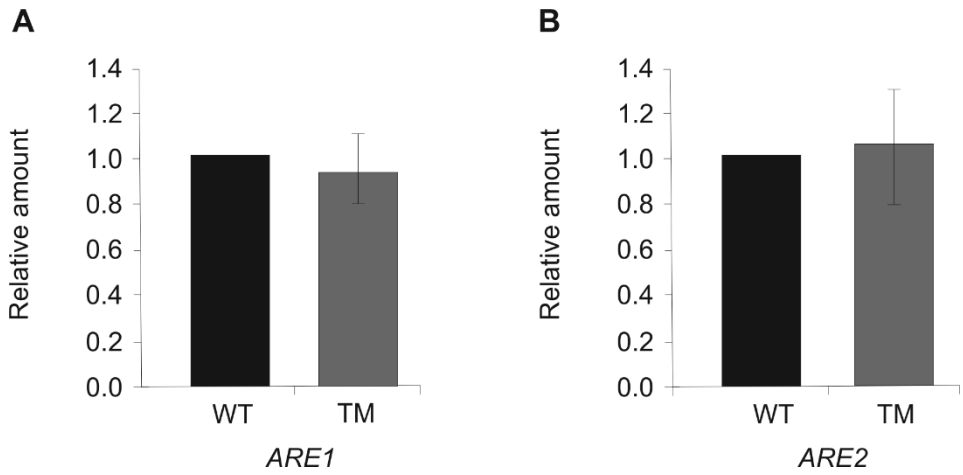

Figure 14. Relative gene expression of the two Acyl-CoA:sterol acyltransferases *ARE1* and *ARE2* in TM (dark grey bars) compared to WT (black bars). Cells were grown to the exponential growth phase, RNA was isolated using the RNeasy kit (Qiagen). Experiments were performed with SuperScript III Platinum SYBR Green One-Step qRT-PCR Kit (Invitrogen) and the expression levels were calculated using the $\Delta\Delta c_t$ method. **A.** Relative gene expression of *ARE1* in TM (mean value 0.9, D 0.2) and WT. **B.** Relative gene expression of *ARE2* in TM (mean value 1.0, D 0.2) and WT. Gene expression in WT was set to 1. Data shown are mean values of at least three independent experiments with the respective deviation as indicated.

5.3. Protein concentration of Are1p and Are2p in a strain lacking steryl ester hydrolases

5.3.1. Amplification of the 13-Myc tag

The finding that transcription of *ARE1* and *ARE2* in the TM was not significantly changed compared to WT led us to investigate the protein levels of the respective gene products. To detect the acyltransferases in WT and TM, it was necessary to add a tag to the proteins of interest. This was done by chromosomal 13-Myc tagging. The Longtine plasmid pFA6a-13Myc-His3MX6 (Figure 15) served as template to amplify the 13-Myc tag.

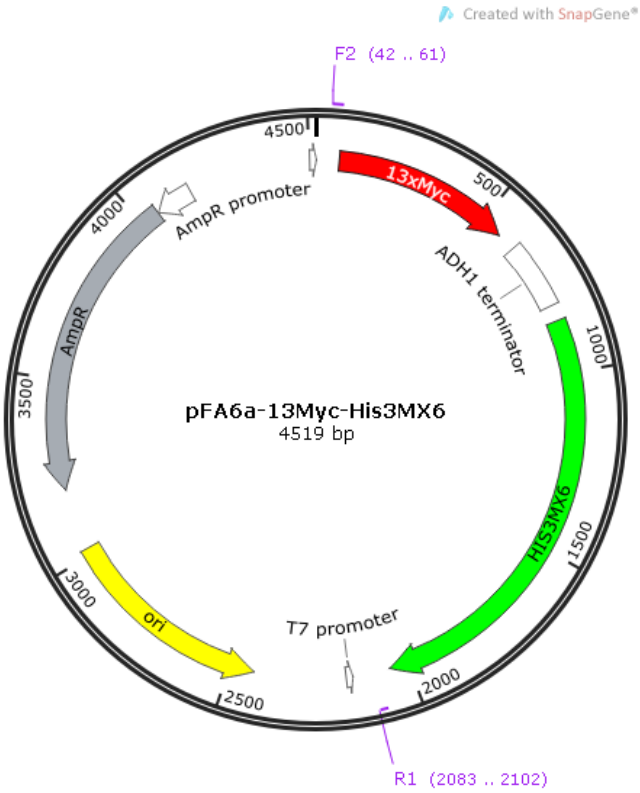

Figure 15. Longtine plasmid pFA6a-13Myc-His3MX6. The 13Myc-tag was amplified to ensure the chromosomal C-terminal myc-tagging of *ARE1* and *ARE2* by homologous recombination. HIS3MX6 served as selection marker to screen for positive clones.

The 2 kbp product was amplified by PCR using primers listed in Table 8. The bands in Figure 16 at the mentioned size indicated the appropriate gene products in a preparative 1% agarose-gel. DNA from lane B and C was pooled and served for tagging *ARE1*, lane D and E represent the DNA for tagging *ARE2*.

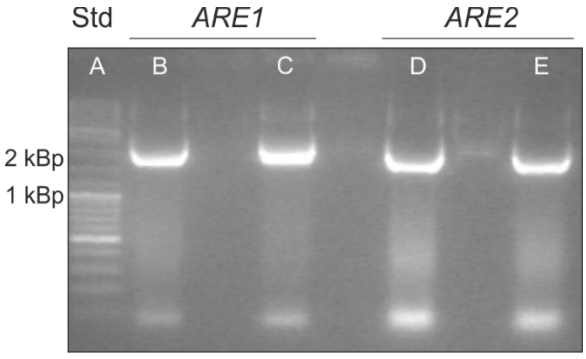

Figure 16. Agarose gel (1%) of products from amplification of the 13-Myc tag from pFA6a-13Myc-His3MX6. The 2 kbp products were cut out and cleaned for homologous recombination. Lane A shows the used standard. Lane B/C: amplified DNA to tag Are1p. Lane D/E: amplified DNA to tag Are2p.

Due to the designed primers, the amplified product contained at 5' ends homologous sequences to the last few nucleotides of *ARE1* and *ARE2*, respectively. The C-terminus was elongated by nucleotides homologous to a small downstream sequence of *ARE1* and *ARE2* (Figure 17).

Figure 17. 2 kbp **c**onstruct after amplification of the 13-Myc tag from the Longtine plasmid pFA6a-13Myc-His3MX6. The hatched part at the 5'end indicates the homologues sequence to the last few nucleotides of *ARE1* and *ARE2*, respectively. The small part following the HIS3MX6 sequence represents the homologous sequence to a small downstream sequence of *ARE1* and *ARE2*, respectively.

This strategy enabled us to make use of chromosomal tagging by homologous recombination. The bands at 2 kbp were cut out of the gel and purified for yeast transformation with the high efficiency lithium acetate transformation protocol [48] as described in *4.1.3*. Therefore, 400 – 700 ng of purified PCR product were used. Homologous recombination yielded the chromosomal tagged genes *ARE1* and *ARE2,* respectively, as shown in Figure 18.

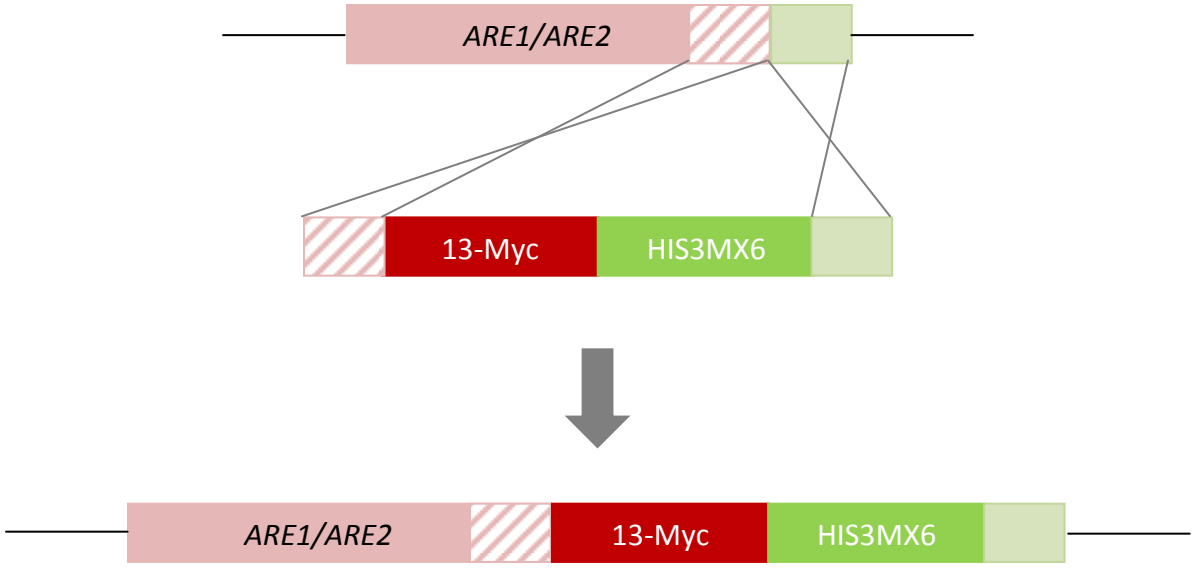

Figure 18. Scheme of homologous recombination.

5.3.2. Colony PCR of positive yeast transformants

To identify positive yeast transformants, cells were plated on SD media lacking histidine, because only positive transformants should be able to produce this essential amino acid and therefore be able to grow. After incubation of agar plates for 2 – 3 days at 30°C, single colonies were picked and subjected to verification by colony PCR.

Colony PCR was carried out as described in section *4.1.1.3* after digestion of cell walls with zymolyase. Forward primers were designed to bind within either *ARE1* or *ARE2*. Reverse primers were homologous to a 13-Myc section.

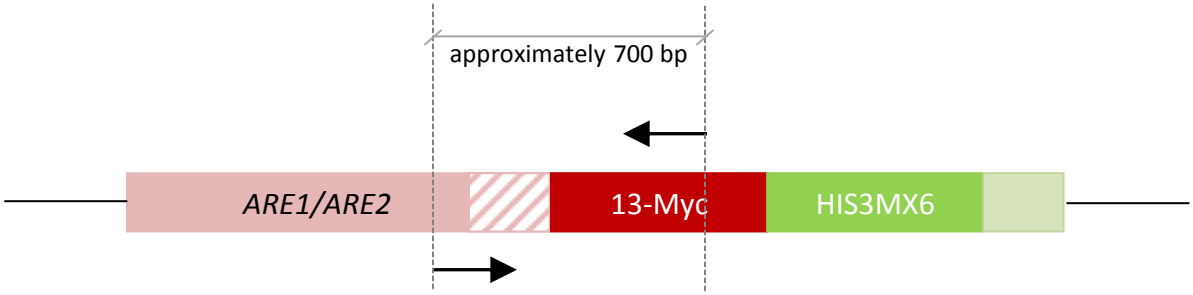

Figure 19. Scheme of tagged *ARE1/ARE2*. The arrows indicate the positions of primers used for colony PCR.

This strategy implies that for a positive transformant in case of *ARE1* a fragment of 697 bp, and for *ARE2* a fragment with the length of 699 bp should be visible in a 1% agarose gel. Figure 20 shows agarose gels with light white DNA bands of positive transformants. Figure B, lane B, and figure C, lane C, show exactly what was assumed for a positive clone, namely one distinct band at the appropriate size. In Figure A, lane B and C, there are some bands representing DNA with smaller size than 700 bp although not at the intensity as the 700 bp band. This is due to the fact that for this PCR the annealing temperature was too low. All cPCRs were done at 55°C except the one in Figure A, where only 50°C were used for primer annealing.

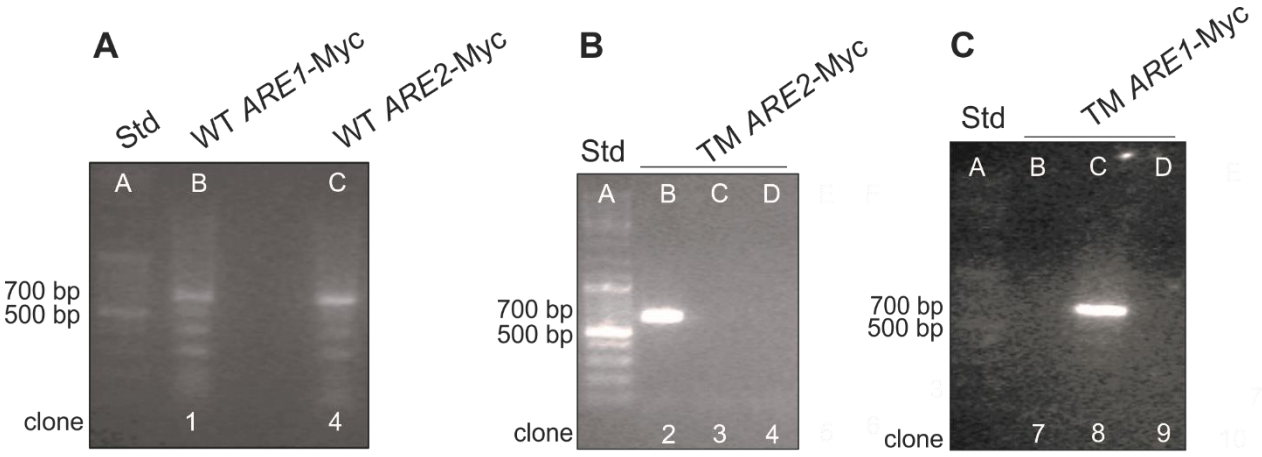

Figure 20. Agarose-gel (1%) from cPCR of positives clones after homologous recombination. Bands of positive clones appear at about 700 bp. Std: standard GeneRuler DNA Ladder Mix. **A.** lane B: WT *ARE1*-Myc, lane C: WT *ARE2*-Myc. **B.** lane B: amplified DNA of a positive transformant TM *ARE2*-Myc **C.** lane C: amplified DNA of a positive transformant TM *ARE1*-Myc.

To verify positive transformants after homologous recombination, several rounds of yeast transformation were necessary. The problem was that either the transformation was not efficient enough to obtain single colonies on the agar plate with SD media lacking histidine, or picked single colonies did not show a band at 700 bp when checked with colony PCR. The latter case could be explained by insertion of the amplified 13-Myc tag and the auxotrophy marker in a wrong position of the DNA. This internal gene conversion of the *his3Δ1* allele of the recipient strain with the *HIS3* marker gene that was introduced was already observed and described by Daniel [61].

5.3.3. Protein levels of Are1p and Are2p tested by Western Blot analysis

According to the Saccharomyces Genome Database SGD (*http://www.yeastgenome.org*) Are2p and especially Are1p are low abundant proteins in *S. cerevisiae*. This fact may explain the noticed difficulties to get comparable signals in Western Blots from homogenates without enrichment of the respective proteins. As both acyltransferases are located to the ER, we isolated microsomes as described in the methods section. The challenge with organelle preparations is, however, to avoid damage of the proteins. To keep protein degradation caused by proteases as low as possible, PMSF was used. After determining the protein concentration of the microsomes from WT and TM, 40 µg protein containing either Are1p or Are2p solubilized in

sample buffer were loaded onto the gel for SDS-PAGE. After blotting, antibody incubation and developing the X-ray film, results as shown in Figure 21 were observed.

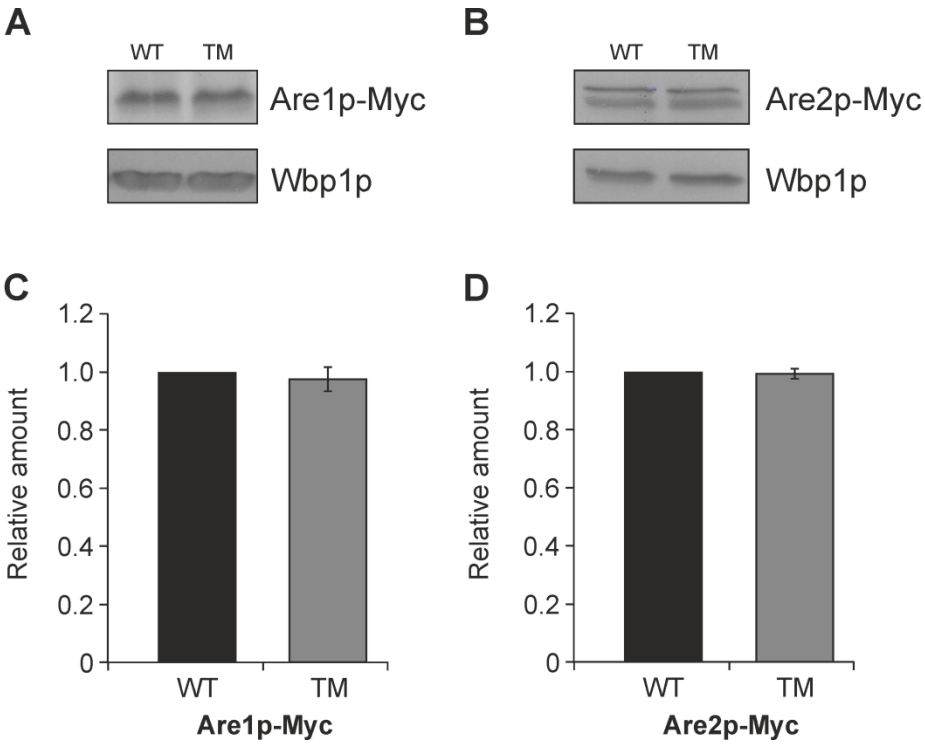

Figure 21. Protein levels of the C-terminal 13-Myc tagged versions of Are1p and Are2p, respectively. Microsomes were prepared, amounts of protein were determined by the method of Lowry [55], and after SDS-PAGE Western Blot analysis was performed. For detection of the 72 kDa and 74 kDa protein of Are1p and Are2p, respectively, mouse anti-Myc antibody (1:1000) and POD conjugated anti-mouse antibody (1:5000) diluted in 2.5% TBST-milk were used. **A.** Bands of Are1p-Myc in TM compared to WT. **B.** Bands of Are2p-Myc in TM compared to WT. Wbp1p served as a loading control. **C.** Calculation of protein amounts by ImageJ referring to A. **D.** Calculation of protein amounts by ImageJ referring to B. Western Blots were done at least from three independent preparations of microsomes. WT (black bars) was set to 1.

Since Wbp1 is an abundant protein in the ER, it served as marker protein and loading control. In both cases, neither Are1p-Myc nor Are2p-Myc showed significant changes of protein amounts in TM compared to WT. Figure B demonstrates that Are2p-Myc appeared in two bands. A possible explanation for this phenomenon could be protein degradation due to still active proteases or mechanical forces. Another explanation may be that proteins underlie posttranslational modifications, e.g. phosphorylation, acetylation or ubiquitinylation. Such modification could result in different running behavior in the gel and appearance of two bands in the Western Blot.

5.3.4. Acyl-CoA:ergosterol acyltransferase assays

As we could not find significant changes at transcriptional and translational levels of *ARE1* and *ARE2* as tested by qRT-PCR and Western Bot, respectively, we tested the enzyme activities of the two acyltransferases. To measure the activity of Are1p and Are2p, acyl-CoA:ergosterol acyltransferase assays with radioactively labeled [^{14}C]oleoyl CoA were performed. To mimic the *in vivo* conditions best, the assays were carried out with untagged versions of Are1p and Are2p. Homogenate and microsomes prepared from WT and TM were used as enzyme sources. As can be seen from Figure 22, the TM exhibited a reduced acyltransferase activity resulting in about half the amount of labeled SE formed compared to WT. This effect was observed with homogenates (A) as well as microsomes (B). These results supported the idea of a possible feedback mechanism.

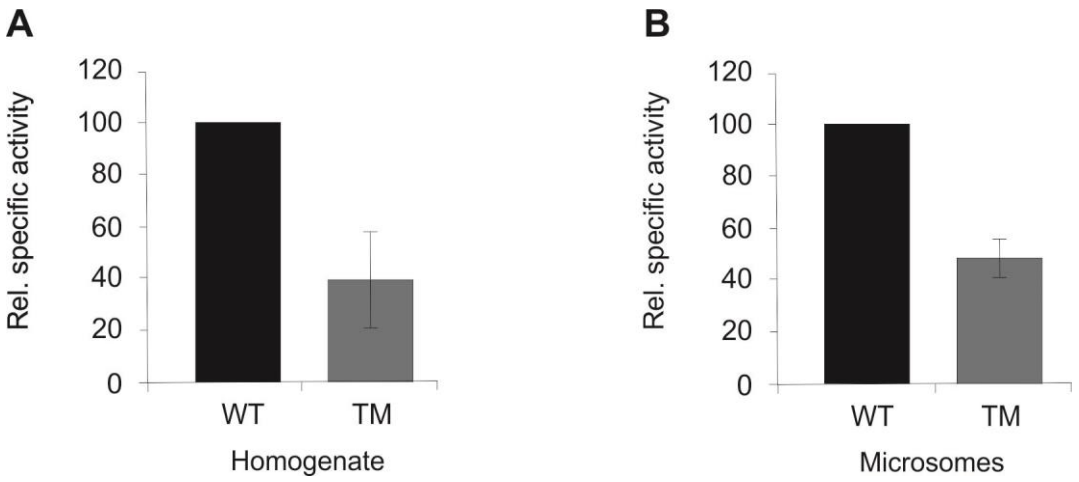

Figure 22. Acyl-CoA:ergosterol acyltransferase assay with untagged Are1p/Are2p in TM compared to WT. **A.** Assay with homogenate (TM in dark grey bar 39%); specific activity of the two *ARE* genes in WT: 1,100 pmol/h/mg. **B.** Assay with microsomes (TM in dark grey bar 49%); specific activity of the two *ARE* genes in WT: 8,300 pmol/h/mg. Solubilized ergosterol, oleoyl-CoA and [^{14}C]oleoyl-CoA were offered as substrates. Reactions were started by adding either 70 µg protein of microsomes, or 250 µg protein of homogenate. Lipids were extracted and separated by TLC, bands were stained with iodine vapor and bands of steryl esters were scraped off. Radioactivity was measured by liquid scintillation counting. Specific activity of WT was set to 100% and relative amounts in TM were calculated. Data shown are mean values of at least three independent experiments performed in duplicates with the respective deviation as indicated.

As acyl-CoA:ergosterol acyltransferase activity was tested initially only in TM and WT, further experiments were performed to elucidate the role of the three individual SE hydrolases Tgl1p, Yeh1p and Yeh2p. The same assays as described above were carried out with the single

deletion mutants of steryl ester SE hydrolases. As shown in Figure 23, this experiment revealed that each of the three SE hydrolases contributed to the reduced activity of Are1p/Are2p although *Δtgl1* showed the most pronounced effect.

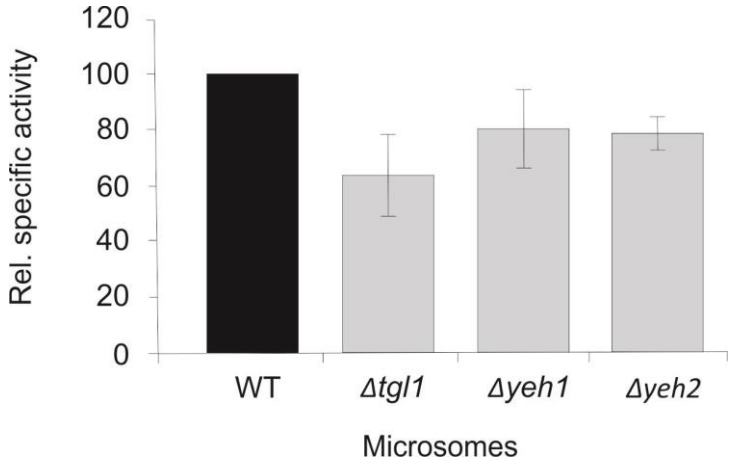

Figure 23. Acyl-CoA:ergosterol acyltransferase assay with microsomes containing untagged Are1p/Are2p from single deletion mutant strains compared to WT. Solubilized ergosterol, oleoyl-CoA and [^{14}C]oleoyl-CoA were offered as substrates. Reactions were started by adding 70 µg protein of microsomes. Lipids were extracted and separated by TLC, bands were stained with iodine vapor and those according to SE were scraped off. Radioactivity was measured by liquid scintillation counting. Specific activity of WT was set to 100% and relative amounts of mutant strains were calculated.. Data shown are mean values of at least three independent experiments performed in duplicates with the respective deviation as indicated.

6. DISCUSSION

Regulation of steryl ester metabolism is an important issue but only poorly understood. Former investigations revealed a heme-dependent regulation of yeast enzymes related to sterol metabolism. Are2p and Yeh2p are the most active acyltransferase and hydrolase, respectively, in aerobic yeast cultures. Under heme-depletion which mimics anaerobic conditions, Are1p acts as the major sterol acyltransferase, and Yeh1p constitutes the main SE hydrolase [27–29]. In addition, Connerth *et al.* [11] showed that elevated levels of oleate acted as competitive enzymatic inhibitors of Are2p causing liposensitivity in yeast.

In this Master Thesis, we investigated the link between SE anabolism and SE degradation. We analyzed the amount of SE in strains bearing defects in SE metabolism. A *Δare1Δare2* strain, which is impaired in synthesizing SE did not show formation of SE (Figure 12). The TM *Δtgl1Δyeh1Δyeh2* lacking all three known SE hydrolases had moderately elevated levels of SE. Interestingly, the *Δtgl1* strain behaved like the TM, whereas *Δyeh1 and Δyeh2* behaved like WT with almost unaffected levels of SE. As mentioned in the introduction, the main SE hydrolase activity *in vitro* was found in the plasma membrane and attributed to Yeh2p [12,31]. It was tempting to speculate that also the amounts of SE in the *Δyeh2* strain are increased. Interestingly, however, a *Δyeh2* strain did not show elevated levels of SE. Analyses performed by B. Ploier (personal communication) showed that the amounts of TG in the TM and in *Δare1Δare2* were increased. As also phospholipids increased to about 130% of wild type in *Δare1Δare2* (Figure 13) we concluded that free fatty acids which are available in excess in the mutant are utilized to build phospholipids and TG instead of SE. In the case of *Δare1Δare2*, results indicated a tight link between non-polar and phospholipid metabolism as shown previously [62,63]. The phospholipid content in the TM, however, was not markedly altered, indicating that there is no link between non-polar and phospholipid metabolism in this mutant strain.

Figure 24 gives an overview of changes in the pattern of non-polar lipids and PL in a strain deleted of the major yeast SE hydrolases.

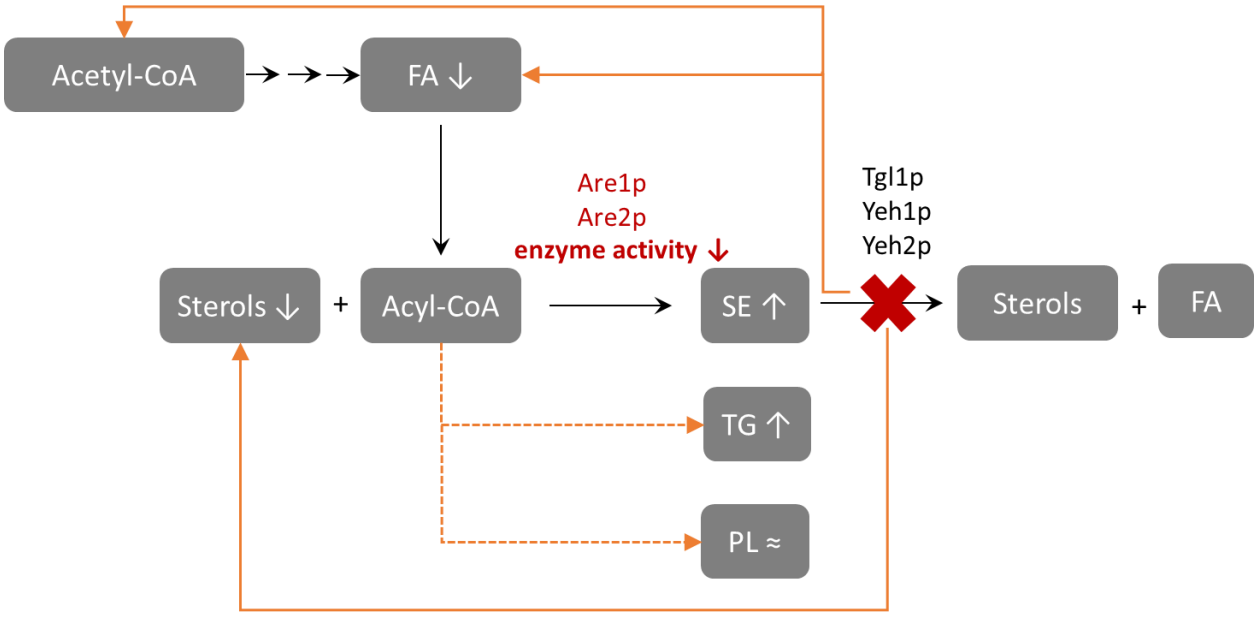

Figure 24. Overview of regulatory effects caused by deletions of *TGL1*, *YEH1* and *YEH2*. Arrows to the top indicate enhanced levels of metabolites (SE, TG) in the TM compared to WT, arrows to the bottom indicate lowered levels of metabolites (FA, sterols). The amounts of PL were not markedly altered.

The fact that in the TM an accumulation of SE was observed, although not as pronounced as expected, led us speculate that a feedback regulatory mechanism to SE formation exists. As one mode of regulation was assumed to occur at the transcriptional level, we investigated the gene expression of *ARE1* and *ARE2* in TM compared to WT. As shown in Figure 14, no significant changes were observed. Thus, we extended our analyses to a possible regulation at the level of proteins. For this purpose, C-terminally Myc tagged Are1p and Are2p from isolated microsomes were quantified. It turned out, that also the amounts of Are1p-Myc and Are2p-Myc were not affected by the block in SE hydrolysis (Figure 21).

Due to the fact, that neither transcription nor translation of SE synthesizing enzymes was altered in the TM, we tested a possible regulation at the enzyme activity level. We hypothesized that reduced enzyme activity of Are1p and Are2p in a strain deficient in hydrolysis of SE might be the reason for the observed changes in the synthesis of non-polar lipids. We performed acyl-CoA:ergosterol acyltransferase assays with isolated microsomes as enzyme source. As can be seen from Figure 22, the TM exhibited a reduced acyltransferase activity

resulting in about half the amount of labeled SE formed compared to WT. This effect was observed with homogenates as well as with microsomes. These results supported the idea of a feedback mechanism of SE hydrolysis to SE formation. To investigate the effect of single deletion mutants in SE hydrolyzing enzymes, the same assay was performed with *Δtgl1, Δyeh1* and *Δyeh2*. These experiments revealed that each of the three steryl ester hydrolases contributed to the reduced activity of Are1p/Are2p, although *Δtgl1* showed the most pronounced effect. As *Δyeh1* and *Δyeh2* strains did not show significant accumulations of SE compared to WT, it was not surprising that activity of the acyltransferases Are1p/Are2p in these single mutants was less than in *Δtgl1*. The effect observed in *Δtgl1* could be explained by a possible accumulation of ergosterol and/or free fatty acids, which may have toxic and therefore inhibiting effects on the acyltransferases. This theory would be in line with findings by Connerth *et al.* [11], who showed that elevated levels of oleate inhibited SE synthesis due to competitive inhibition of Are2p. Another explanation could be that deletion of one of the SE hydrolases might have effects either on transcription, translation or activity of enzymes involved in the ergosterol biosynthesis pathway. In case of a down regulation of enzymes responsible for ergosterol biosynthesis, a substrate limitation of ergosterol might cause the reduced activities of Are1p/Are2p. Vice versa, the accumulation of ergosterol by up regulation of the sterol biosynthesis pathway could show toxic effects on the acyltransferases resulting again in a reduced activity. The effect may also be applicable to free fatty acids and enzymes involved to their synthesis.

In recent experiments (B. Ploier; personal communication), a possible feedback regulation to SE synthesizing enzymes in the TM was explained by substrate limitation *in vivo*. Amounts of sterols and fatty acids in wild type and TM were analyzed by GC/MS. A reduced formation of total cellular sterols to about 65% in the TM compared to WT was shown. Free sterols in the TM were also markedly reduced, which could be explained by a slight down regulation of *ERG2* and *ERG6*. Similarly, the amount of fatty acids in the TM was decreased to about 75% compared to WT. These results supported a possible feedback mechanism of SE hydrolysis to SE formation due to substrate limitation of sterols and fatty acids. In these studies incorporation of [^{14}C]oleic acid into PL, TG and SE *in vivo* were measured. It was demonstrated that the TM deficient in all three SE hydrolases incorporated the same amount of [^{14}C]oleic acid into phospholipids as WT.

The amount of label in TG was markedly increased in the TM, whereas reduced incorporation of the label in SE was measured. *In vivo* labeling with [^{14}C]acetic acid also covered total fatty acid biosynthesis. Also the formation of fatty acids in the TM was markedly reduced as compared to WT. Again, decreased incorporation of the label into SE was found in the TM compared to WT confirming lowered activity of Are1p/Are2p in the mutant.

Altogether, work performed in this Thesis showed clearly a regulatory link between SE hydrolysis and synthesis. The further upstream regulation of sterol and fatty acid synthesis may be regarded as broader effect on the lipid regulation network. Despite the insight in the regulation of SE metabolism obtained in this study, a number of questions remained open. Interesting experiments concerning the regulation of SE metabolism may be, for example, quantification of transcription, protein and enzyme activity levels of Tgl1p, Yeh1p and Yeh2p in a *Δare1Δare2* strain. Such experiments may help to understand the behavior of the SE hydrolases in a strain deficient in building SE synthesis. Further links to the synthesis and the turnover of all lipids may also be worth testing.

7. APPENDIX

7.1. Table of figures

7.2. List of tables

8. ABBREVIATIONS

APS	ammoniumpersulfat
bp	base pairs
BSA	bovine serum albumin
cww	cell wet weight
cPCR	Colony PCR
DEPC	diethyl pyrocarbonate
DG	diacylglycerol
dH_2O	deionized water
D	standard deviation
DMSO	dimethyl sulfoxide
DNA	desoxyribonucleic acid
dNTP	desoxynucleotid triphosphate
DTT	dithiothreitol
EDTA	ethylendiamintetraacetate
ER	endoplasmic reticulum
FA	fatty acids
FFA	free fatty acids
kBp	kilo base pairs
kDa	kilo Dalton
LB	lysogeny broth medium
LiAc	lithium acetate
LD	lipid droplet
OD_{600}	extinction at 600 nm
ONC	overnight culture
ORF	open reading frame
PAGE	polyacrylamide gel electrophoresis
PCR	polymerase chain reaction
PEG	polyethylene glycol
PL	phospholipids
RT	room temperature
SD	synthetic dropout medium (minimal medium)

SD all	SD with all amino acids supplemented
SDS	sodium dodecyl sulfate
SE	steryl ester
TG	triacylglycerols
TCA	trichloroacetic acid
TEMED	N,N,N',N',-tetramethyl ethylene diamide
TLC	thin layer chromatography
YPD	yeast peptone dextrose medium

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