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Eukaryotic sterol homeostasis: Steryl ester hydrolases in *Saccharomyces cerevisiae*

DISSERTATION

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Table of contents

Summary

Sterol homeostasis in eukaryotic cells relies on the reciprocal interconversion of free sterols and steryl esters (STE). The formation of STE is well characterized, but the mechanisms that control steryl ester mobilization upon cellular demand are less well understood. STE constitute an important storage form for fatty acids and sterols that are deposited in intracellular lipid particles. To identify genes that are required for the mobilization of STE, we performed an *in silico* analysis of the yeast genome to identify putative STE hydrolase encoding genes. This candidate gene approach revealed eight putative lipase encoding genes. To test whether one or more of these putative lipases are required for STE hydrolysis we developed *in vivo* assays to monitor hydrolysis of radiolabeled steryl esters in *Saccharomyces cerevisiae*. Upon depletion of endogenous sterol biosynthesis by terbinafine, hydrolysis of [^{3}H]palmitic acid- or [^{14}C]cholesterol-labeled steryl esters was monitored in cells bearing deletion of candidate hydrolase genes. This analysis revealed that the rate of STE mobilization, but not that of triacylglyerol (TAG), is strongly decreased in cells lacking *YLL012*/*YEH1* (ster*y*l *e*ster *h*ydrolase 1), *YLR020/YEH2*, or *TGL1*. These lipases are paralogues of the mammalian acid lipase family, which is composed of the lysosomal acid lipase, the gastric lipase, and four novel as yet uncharacterized human open reading frames. Lipase single mutants mobilize STE to various degrees, indicating partial functional redundancy of the three gene products. A triple *yeh1Δ yeh2Δ tgl1Δ* mutant shows no STE mobilization, indicating that these three genes together encode for all the STE hydrolyase activity present in yeast. STE hydrolase activity of the three lipases was confirmed by *in vitro* assays with radiolabeled cholesteryl ester as substrate. Functional GFP-tagged Yeh1p and Tgl1p co-localize with Erg6p, a marker protein for lipid particles, and are enriched in isolated lipid particles. Interestingly, the third lipase, Yeh2p, is localized to the cell periphery and is absent from lipid particles. *YEH1*, *YEH2*, and *TGL1* encode predicted membrane proteins and were shown to be solubilized by detergent treatment only, indicating that they indeed behave as integral membrane proteins. This makes them the first membrane-anchored lipases described so far. Proteinase protection experiments, using GFP-tagged lipases, enabled us to also determine their membrane topology. The mechanisms that control STE mobilization are not well understood. The mammalian hormone sensitive lipase (HSL) has been shown to be phosphorylated upon lipolytic stimulation, which resulted in a 100-fold increase of its activity. Detailed analysis of the three STE hydrolases revealed that Yeh2p, but not Yeh1p or Tgl1p, is modified by phosphorylation and this phosphorylation depends on the growth phase of the cell. A deletion mutant screen for protein kinases that are required for phosphorylation

of Yeh2p revealed that every one of the four protein kinases, *SWE1*, *VHS1*, *KCC4*, and *YNR047* is required for phosphorylation of Yeh2p. The biological significance of this posttranslational modification, however, is not yet known. Yeast is a facultative anaerobic organism that becomes auxotrophic for sterols and unsaturated fatty acids in the absence of oxygen. It is also reported here that Yeh1p is the only active STE hydrolase in heme-deficient conditions. The steady-state levels of Yeh1p are significantly increased in heme-deficient cells, which is in line with the observation that Yeh1p is important for STE mobilization under these conditions.

Taken together, the work presented here describes a novel class of membrane-anchored lipases required for STE mobilization in yeast. These lipases differ in their subcellular localization and membrane topology. Future studies based on the data acquired here will allow to provide detailed insight into regulation of the three lipases and also help to clarify the physiological importance of STE in yeast. The question how STE storage and mobilization is regulated in humans is important for the understanding of an increasing number of human disorders in lipid metabolism, such as atherosclerosis, obesity and type 2 diabetes. The three STE hydrolases described in this work are paralogues of the mammalian acid lipase family and yeast will thus provide an excellent model organism to address the question how STE mobilization is regulated which in turn should be valuable for the understanding of basic principles of STE homeostasis in mammals.

Zusammenfassung

Sterol-Homeostase in Eukaryonten beruht auf der reziproken Umwandlung von freien Sterolen und Sterol-Estern (STE). Die Mechanismen des Aufbaus von STE sind bekannt, diejenigen der Mobilisierung von STE sind jedoch noch weitgehend unbekannt. STE stellen eine wichtige Speicherform für Fettsäuren und Sterole dar und werden intrazellulär in Lipidpartikeln gespeichert. Um die Enzyme, welche für die Mobilisierung von STE notwendig sind, zu identifizieren, führten wir eine *in silico*-Analyse des Hefegenoms zur Auffindung von für STE-Hydrolasen kodierenden Genen durch. Die Genomanalyse zeigte acht potentielle STE-Hydrolase kodierende Gene. Um zu testen, ob eine oder mehrere dieser Lipasen für die Mobilisierung von STE notwendig sind, entwickelten wir einen *in vivo*-Assay, mit welchem wir die Hydrolyse von radioaktiv markierten STE in *Saccharomyces cerevisiae* beobachten konnten. In Mutanten, welche für Lipase-Kandidaten Gene deletiert waren, wurde durch Blockierung der endogenen Sterol-Biosynthese mit Terbinafine die STE Mobilisierung initiiert. Dies ermöglichte uns die Rate der Hydrolyse von [^{3}H]Palmitinsäure oder [^{14}C]Cholesterol-markierten STE in diesen Mutanten zu bestimmen. Diese Experimente zeigten, dass die Rate der STE-Mobilisierung in Zellen, die für *YLL012/YEH1* (Steryl-*E*ster-*H*ydrolase *1*), *YLR020/YEH2* oder *TGL1* deletiert waren, sehr gering war. Dies traf jedoch nicht auf die Hydrolyse von Triacylglycerol (TAG) zu. Diese drei Lipasen sind Paraloge der menschlichen sauren Lipasefamilie, welche aus der lysosomalen sauren Lipase, der gastrischen Lipase und vier neuen, bisher nicht charakterisierten menschlichen ORFs besteht. Lipasemutanten mobilisieren STE in verschiedenem Ausmaß, was durch eine teilweise funktionelle Überlappung der Aktivität der drei Genprodukte bedingt ist. Eine *yeh1Δ yeh2Δ tgl1Δ*-Triple-Mutante hingegen zeigt keine STE-Mobilisierung mehr, was darauf schließen lässt, dass die drei Genprodukte zusammen für sämtliche STE-Hydrolase-Aktivitäten in Hefe verantwortlich sind. Die STE-Hydrolase-Aktivität der drei Lipasen wurde durch *in vitro*-Assays, in denen radioaktiv markierte Cholesteryl-Ester als Substrat verwendet wurden, bestätigt. Funktionelle GFP-markierte STE-Hydrolasen Yeh1p und Tgl1p kolokalisieren mit Erg6p, einem Markerprotein für Lipidpartikel, und sind in isolierten Lipidpartikeln angereichert. Interessanterweise lokalisiert die dritte Lipase, Yeh2p, an der Zellperipherie und kann nicht in Lipidpartikeln gefunden werden. Laut Sequenzanalysen kodieren *YEH1*, *YEH2* und *TGL1* für Membranproteine. Die drei Lipasen können nur in Gegenwart von Detergenz solubilisiert werden, was darauf schließen lässt, dass sie sich tatsächlich wie integrale Membranproteine verhalten. Dadurch sind diese die ersten Membran-verankerten Lipasen, die

bisher beschrieben wurden. Proteinase-Verdau-Experimente, in denen GFP-markierte Lipasen benutzt wurden, ließen uns auch die Membrantopologie der drei Lipasen feststellen.

Die Mechanismen, welche die STE-Mobilisierung kontrollieren, sind noch weitgehend unbekannt. Beispielweise wird die menschliche hormonsensitive Lipase (HSL) nach lipolytischer Stimulation phosphoriliert, was in einer hundertfachen Aktivitätssteigerung resultiert. Eine detaillierte Analyse der drei STE-Hydrolasen zeigte, dass zwar Yeh2p durch Phosphorilierung modifiziert wird, nicht aber Yeh1p oder Tgl1p. Diese Phosphorilierung hängt von der Wachstumsphase der Zelle ab. Ein Mutantenscreen nach Proteinkinasen, welche für die Phosphorilierung von Yeh2p nötig sind, zeigte, dass jede der vier Proteinkinasen, *SWE1*, *VHS1*, *KCC4* und *YNR047* für die Phosphorilierung von Yeh2p notwendig ist. Die biologische Signifikanz dieser posttranslationalen Modifikation ist bisher noch nicht bekannt. Die Hefe ist ein fakultativ anaerober Organismus, der in Abwesenheit von Sauerstoff auxotroph für Sterole und ungesättigte Fettsäuren wird. Es wird in dieser Arbeit auch gezeigt, dass Yeh1p die einzige aktive STE-Hydrolase unter Häm-depletierten Konditionen ist. Die “steady-state-levels“ von Yeh1p sind in Häm-depletierten Zellen signifikant erhöht, was mit der Beobachtung kongruiert, dass Yeh1p wichtig für STE-Mobilisierung unter diesen Bedingungen ist.

Zusammengefasst bedeutet dies, dass die hier präsentierte Arbeit eine neue Klasse von Membran-verankerten Lipasen, die für die Mobilisierung von STE in Hefe notwendig sind, beschreibt. Diese Lipasen unterscheiden sich in ihrer subzellulären Lokalisation und der Membrantopologie. Zukünftige Studien, die sich auf das hier erarbeitete Material stützen, könnten detaillierte Einblicke in die Regulation der drei Hefelipasen geben und dazu beitragen, die physiologischen Aspekte von STE in Hefe zu klären. Da man erst beginnt, die regulatorischen Aspekte von STE-Lagerung und -Mobilisierung im Menschen zu verstehen, besteht ein großes Interesse an der Regulierung und vor allem an den Konsequenzen von Defekten im Neutral-Lipid-Metabolismus. Heutzutage steigt die Anzahl schwerer Krankheiten, verursacht durch Lipid-Akkumulation, wie z.B. Arteriosklerose, Adipositas und Typ-2-Diabetes, stetig an. Die drei neu entdeckten und hier beschriebenen STE-Hydrolasen sind Paraloge der menschlichen sauren Lipasefamilie. Somit stellt die Hefe einen exzellenten Modellorganismus dar, der wichtige Fragen betreffend die Regulation von STE-Mobilisierung klären kann. Die Ergebnisse sollten zu einem besseren Verständnis der grundsätzlichen Mechanismen der Cholesterol-Homeostase in Menschen führen.

CHAPTER I

Eukaryotic sterol homeostasis

Introduction

1. Introduction

Sterols are essential lipids of eukaryotic cells. The principal fungal sterol is ergosterol which differs from cholesterol only by the presence of additional double bonds at C7 and C22 and a methyl group at C28 (Fig.1). Apart from that, the structure of cholesterol and ergosterol is identical and both sterols exhibit similar cellular functions. The biosynthesis of ergosterol is also very similar to that of cholesterol of mammalian cells and most of the components of the biosynthetic machinery are well conserved between fungi, plants, and mammals (1, 2). Biosynthesis of sterols is a very energy demanding process, which makes them an extremely metabolically costly component (3). The energy demanding process of sterol biosynthesis must be justified by its function. Free sterols are orientated with the hydroxyl group towards the aqueous environment and are found in all cellular membranes like the ER, the Golgi apparatus, but are greatly enriched in the plasma membrane (4). Over 90% of the free sterol is located in the plasma membrane. Free sterols in membranes are orientated with the hydroxyl group towards the aqueous environment. In addition to the free sterols that are found in the membranes there is a second major form of sterols, steryl ester (STE). STE are often called extramembraneous lipids since they constitute a storage form of sterols and are excluded from membranes.

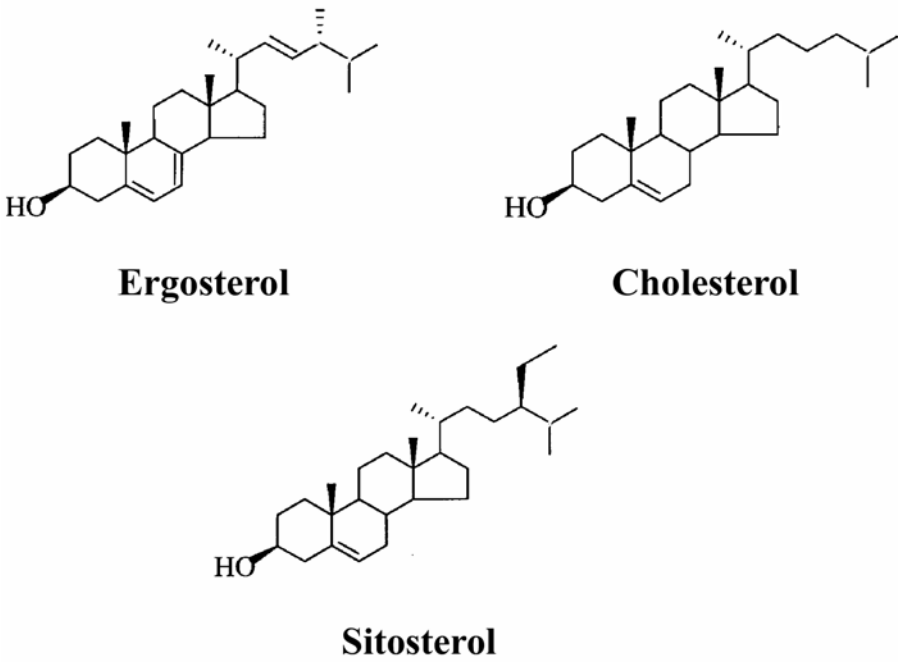

Figure 1: Structure of the principal membrane sterols in fungi (ergosterol), mammals (cholesterol), and plants (sitosterol).

1.1. Sterol homeostasis

For an overview of eukaryotic sterol homeostasis see Fig.2 (detailed description in the following text). STE are synthesized in the ER and are composed of a long chain fatty acid which is esterified to the 3-beta OH-group of the sterol or sterol precursors by acyl-CoA-cholesterol acyltransferases (ACAT) (Fig.3). The STE pool is thought to be important as it serves to buffer an excess as well as a lack of free sterols (6). The free sterols and the STE pools undergo dynamic changes and interconversion, which are tightly linked to the cell cycle (9). It has also been shown that the free sterol pool remains relatively constant, whereas the level of STE is very low during logarithmic growth and increases dramatically when cells enter the stationary phase (8). When stationary cells exit this growth phase, e.g. when they are diluted into fresh media and start to grow again, the STE pool is rapidly mobilized and STE are hydrolyzed to free sterols and fatty acids. The free sterols can then be used for membrane biogenesis (9). In addition to the storage lipid function of STEs another important aspect of STE formation was proposed. This second important function of the sterol esterification process is called “proofing function”. This proofing function is defined as the removal of non-ergosterol sterols or less desirable sterols (e.g. biosynthetic precursors) from the membrane forming pool of free sterols. Esterification of zymosterol, lanosterol, fecosterol, and episterol would thus exclude these precursors from being incorporated into membranes, which might be detrimental. Incorporation of such sterol precursors into the plasma membrane of yeast *erg* mutants was shown to alter the physiological properties of the membrane (5). This would explain why large amounts of zymosterol, lanosterol, fecosterol, and episterol are esterified to fatty acids, because this may protects the cell from accumulation of these less desirable sterols (10, 11, 12). Moreover, an excess of ergosterol from overproduction may also be detrimental for normal membrane function and could be removed and “neutralized” by esterification and stored for later demand (3). This could also be seen as a detoxification mechanism. For example cholesterol is very rapidly and efficiently esterified and might in this way be removed from the endogenous free ergosterol pool, when fed to yeast cells. This removal of a non-ergosterol sterol could help to protect the cell from toxicity of an excess of cholesterol, because cholesterol might not be able to fulfil all the functions of the fungal sterol ergosterol in yeast (13, 7). Taken together, sterol homeostasis and its control is of great importance for the cell since sterols are essential lipids of membranes but also detrimental in excess and metabolically very expensive compounds.

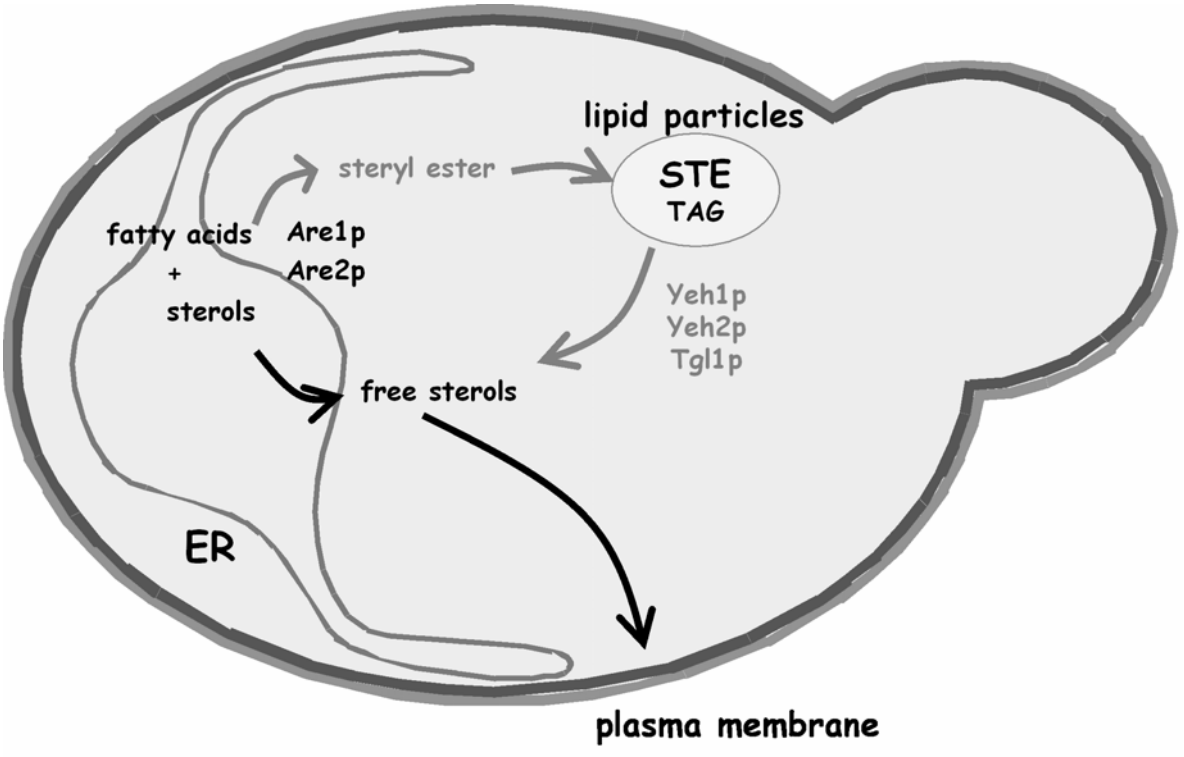

Figure 2: An overview of eukaryotic sterol homeostasis in *Saccharomyces cerevisiae*. STE are synthesized by Are1p and Are2p in the ER and deposited in lipid particles for later demand. Hydrolysis of STE is carried out by the action of three STE hydrolase, namely Yeh1p, Yeh2p, and Tgl1p, to release free sterols are then incorporated into membranes again. STE, steryl ester; TAG, triacylglycerol. For a detailed description see text.

1.1.1. Synthesis of steryl ester

The formation of STE is catalyzed by acyl-CoA-cholesterol acyltransferases which esterify sterols in an Acetyl-CoA dependent fashion by adding a free fatty acid to the 3-OH group of sterols (Fig.3). In yeast there exist two ACAT related genes, *ARE1* and *ARE2*, which together constitute for all the STE activity (14, 15). The two yeast genes, *ARE1* and *ARE2*, exhibit high homology of around 20% to the human *ACAT*, which was identified much earlier (16). In line with the evolutionary conservation of ACAT family genes, the human *ACAT* is able to complement the STE synthesis defect of an *are1Δ are2Δ* double mutant (15). Furthermore, *ARE1* and *ARE2* are themselves highly related to each other (49% identity). Both enzymes are localized to the ER as demonstrated by enzymatic assays and protein localization studies (17, 18). Deletion of both *ARE1* and *ARE2* results in a complete loss of STE synthesis although the cells remain viable, which shows that STE synthesis is non essential under standard growth conditions (14). Due to the presence of two ACATs in yeast, it was suggested that the two enzymes exhibit different substrate specificity with respect to the sterol moiety in the STE. Indeed, Are2p exhibits preference for ergosterol as a substrate, whereas Are1p mainly esterifies lanosterol and other sterol precursors.

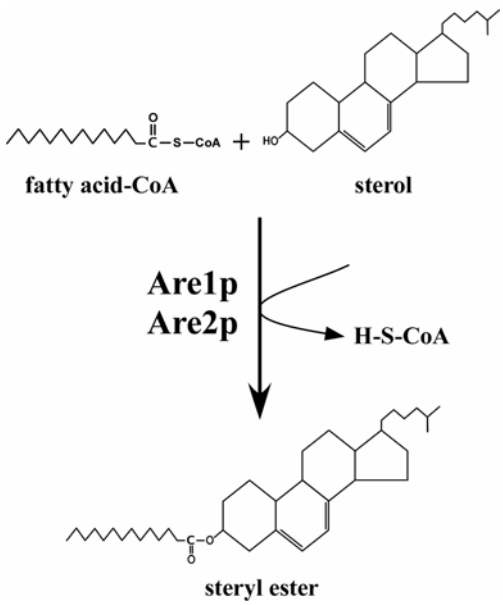

Figure 3: STE formation by the steryl ester synthases Are1p and Are2p in yeast.

Interestingly, no specificity of the enzymes towards the fatty acids could be observed. The major fatty acids in STE are C16:1 and C18:1, whereas saturated fatty acids like C14:0, C16:0 and C18:0 are incorporated less efficiently (18). In addition to the different substrate specificity of Are1p and Are2p, the two genes are also expressed differently (19). Experiments using *ARE1* and *ARE2* promotor fusions to lacZ reporters showed that transcriptional initiation from the *ARE2* promotor is significantly higher/increased compared to the *ARE1* promotor. In other words, *ARE2* seems to be the main STE synthase in yeast and this is consistent with the observation that under normal growth conditions the contribution of *ARE2* to the total STE mass accounts for about 75% (14). Another important finding explaining the contribution of *ARE1* and *ARE2* in STE synthesis came from the work of the laboratory of Ivan Hapala. They showed complex changes in the sterol esterification pattern during anaerobiosis in yeast and confirmed that Are2p exhibits its major activity in aerobic cells. In contrast, Are1p has higher activity under anaerobic conditions (21). Since yeast is a facultative anaerobic organism, these results partially explain the presence of two STE synthases. The beneficial effect of high activity of Are1p under anaerobic conditions becomes evident when one considers that ergosterol biosynthesis requires oxygen. Thus, in oxygen depleted cells sterol precursors should accumulate and would be esterified preferentially by Are1p. Moreover, expression of the two yeast acyl-CoA:sterol acyltransferases *ARE1* and *ARE2* is regulated by heme (22). Heme itself is required as a cofactor for various steps in ergosterol biosynthesis and there are also effects of the cellular heme status on exogenous sterol uptake and esterification (23). Furthermore, the pattern of sterol esterification typical

for anaerobic cells can be mimicked by heme deficiency during aerobiosis. These changes in sterol esterification by the heme status thus are the result of different transcriptional regulation and substrate preference of *ARE1* and *ARE2* (22).

Taken together, the presence of two isoenzymes for STE synthesis preferentially utilizing different sterols and their different regulation in anaerobiosis and aerobiosis clearly shows the importance of STE synthesis as being part of a poorly defined pathway that regulates cellular sterol homeostasis. Moreover, this might describes the importance and different function of sterol esterification under conditions optimal for ergosterol biosynthesis (Are2p and aerobic conditions) and esterification of sterol precursors e.g. lanosterol or non-natural sterols (Are1p and anaerobic conditions) in hypoxic cells.

1.1.2. Intracellular lipid particles

Lipid particles or lipid droplets are the intracellular storage organelle for neutral lipids and can be found in all types of eukaryotes like plants (24), mammals (25), yeast (26) and even in some prokaryotes (27, 28). The structure of lipid particles is rather simple and conserved in all kingdoms of life. They consist of a highly hydrophobic core of neutral lipids, mainly TAG and STE and are surrounded by phospholipids (Fig.4). Lipid particles thus serve as a storage organelle for energy and components required for membrane biogenesis. In addition, lipid particles often contain proteins of different biosynthetic pathways that are mostly involved in lipid metabolism and therefore might also participate in cellular processes that are not directly linked with the storage function of this organelle. For example in yeast three enzymes of the ergosterol biosynthetic pathway, Erg1p, Erg6p and Erg7p, are localized to lipid particles, which suggest a possible function of lipid particles not only in storage of neutral lipids but, maybe also in lipid biosynthesis (29, 30, 31).

1.1.2.1. Lipid droplets of mammalian cells and plants

Lipid droplets of mammalian cells and oil bodies of plants share similar structure with variations in the composition of the hydrophobic core. The neutral lipid composition of lipid droplets of mammalian cells varies within different tissues. For example in bovine heart muscle the core of the lipid droplets consists mainly of TAG (~95%) whereas in stellate cells of the liver the hydrophobic core consists mainly of retinyl ester and TAG (42% and 28%) (25, 33). Lipid droplets with high levels of STE and small amounts of TAG can be found primarily in steriodogenic cells but also in atherosclerotic lesions and macrophage foam cells (34, 35, 36). In plant cells lipid droplets which are often called oil bodies mainly serve as a

store for TAG (95%) and to a minor extent DAG (4%) (37, 38). STE are only minor represented in oil bodies, although lipid droplets from maize and celery contain higher amounts of STE (39). Both lipid droplets in mammalian cells as well as oil bodies in plants contain a specific set of associated proteins. A major protein of mammalian lipid droplets is perilipin and adipose differentiation related protein (ADRP). Perilipins are important for lipid droplet formation and lipolysis (40, 41). They belong to a family of proteins which are phosphorylated upon hormone stimulus and are localized to the periphery of lipid droplets. Similar to perilipins in mammals, the most prominent proteins of plant oil bodies are the oleosins, which are also associated with the surface of the oil bodies and seem to function like perilipins (42).

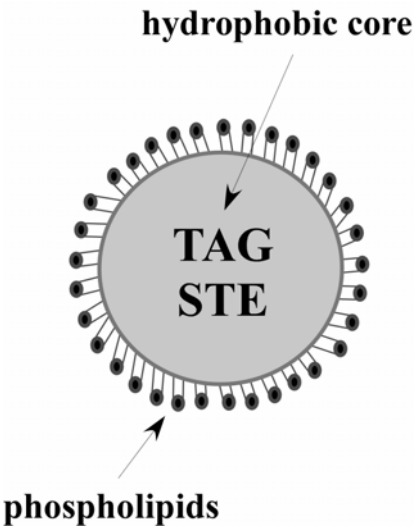

Figure 4: Schematic representation of a lipid particle in yeast. STE, steryl ester; TAG, triacylglycerol.

1.1.2.2. Lipid particles of yeast

Lipid particles of yeast are also composed of a hydrophobic core of neutral lipids which is surrounded by a phospholipid monolayer. The hydrophobic core is composed of nearly equal amounts of STE and TAG (~50% each) (26, 30, 17). Like their counterparts in higher eukaryotes, lipid particles of yeast also contain a set of associated protein. Studies by Athenstaedt *et al.* identified 16 major yeast lipid particle proteins by systematic mass spectrometry analysis (43). Most of these proteins are required for lipid metabolism, such as Erg1p, Erg6p, Erg7p, Faa1p, and Faa4p (44, 30, 45, 46, 47). Interestingly, no proteins with homology to the mammalian perilipin family or the plant oleosin family are present in yeast.

Recently, several lipolytic enzymes were found to be associated with lipid particles. Three TAG lipases encoded by the genes *TGL3*, *TGL4*, and *TGL5* as well as the STE hydrolases Yeh1p and Tgl1p were found exclusively associated with lipid particles (48, 49, 50, 51). Taken together, the set of proteins located and/or associated with lipid particles, such as

enzymes of the ergosterol biosynthetic pathway or neutral lipid lipases, show that lipid particles might not only serve as a storage organelle in yeast, but also participate in lipid metabolism.

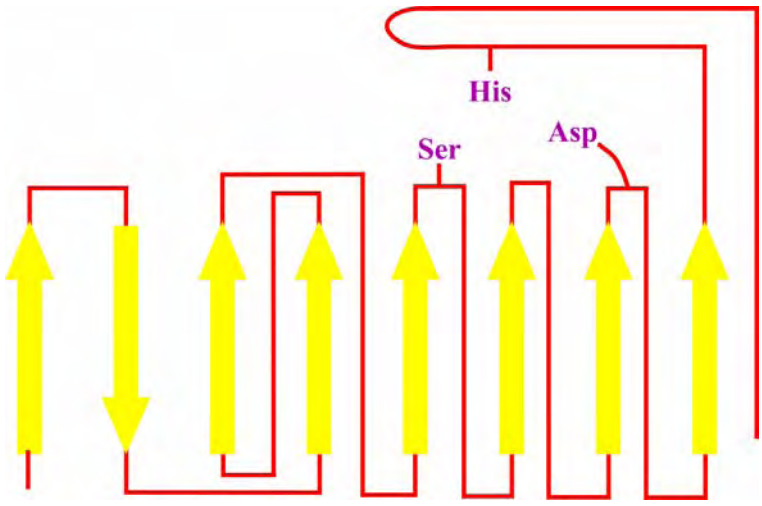

Figure 5: Schematic representation of the alpha/beta hydrolase fold. Eight beta-sheets (yellow) are connected by helical loops (red). The amino acid residues of the catalytic triad composed of Ser, His, and Asp are shown in purple.

2. Steryl ester hydrolases

2.1. Lipases and esterases – the alpha / beta hydrolase family

Lipolytic enzymes are currently attracting a lot of attention, not only because of their biotechnological potential, but also because of their biological importance in lipid metabolism and lipid associated diseases, such as atherosclerosis and obesity (52, 53). This class of enzymes includes carboxylic acid ester hydrolases, lipid hydrolases, thioester hydrolases, peptide hydrolases, haloperoxidases, haloalkane dehalogenases, and epoxide hydrolases. One can distinguish different classes of lipolytic enzymes which exhibit various activities against different substrates. Carboxylesterases hydrolyze partially water soluble ester substrates and in contrast the “real” lipases display also activity towards substrates such as TAG and STE and act at a lipid-water interface. Another class of lipolytic enzymes is composed of phospholipases, which will not be discussed here (for further information on phospholipases see Ref. 54). The largest group of lipases is the so-called alpha/beta hydrolase family, named according to the structural architecture. This superfamily of enzymes includes a variety of enzymes with a broad range of substrates and functions (55, 56). As the name of the alpha/beta hydrolase superfamily already suggests, their three dimensional structure reveals the characteristics of a so-called alpha/beta hydrolase fold which compromises eight beta

sheets connected by helical loops (Fig.5, Fig.6) (57, 55, 59). In addition, a catalytical triad composed of the three amino acids Ser, Asp (or Glu; a carboxylic acid residue) and His can be found, where the active Ser itself is part of a consensus sequence Gly-X-Ser-X-Gly. A lot of attention was drawn to true lipases. Many members of this enzyme family contain loop insertions that fold into sub-domains on the carboxyl edge of the principal sheet. These secondary structure elements form the so-called lid, which is important for regulated access to the substrate binding and active site of the enzymes. Their preference for water insoluble substrates and their absorption at the oil water interface before hydrolysis involves substantial changes in the conformation of the enzyme during binding and catalysis (60, 61). Another structural motif is the nucleophilic elbow which compromises the catalytical triad residues in a tight turn on the fifth beta strand and the following helix. The nucleophilic elbow allows efficient presentation of the nucleophilic Ser at the site of attack on the substrate. The catalytical triad is well conserved in its position between family members. One more important feature of the active site of alpha/beta hydrolases is the spatial arrangement of hydrogen bond donors within the so-called oxyanion hole (62). The hydrolysis reaction takes place as follows. The carboxyl carbon of the released fatty acid is covalently bound to the nucleophilic Ser during the reaction and the His residue of the catalytical triad donates a hydrogen to the released alcohol. A water molecule is then hydrolyzed and added to the carboxyl group and in parallel the Ser regains its hydroxyl group. The oxyanion hole stabilizes the anionic transition state of the substrate by two or three hydrogen bonds**.** Taken together**,** assisted by the oxyanion hole, the three amino acids of the catalytical triad catalyzes the hydrolysis of the ester bond by stabilizing the charge distribution of the transition states during substrate catalysis (63).

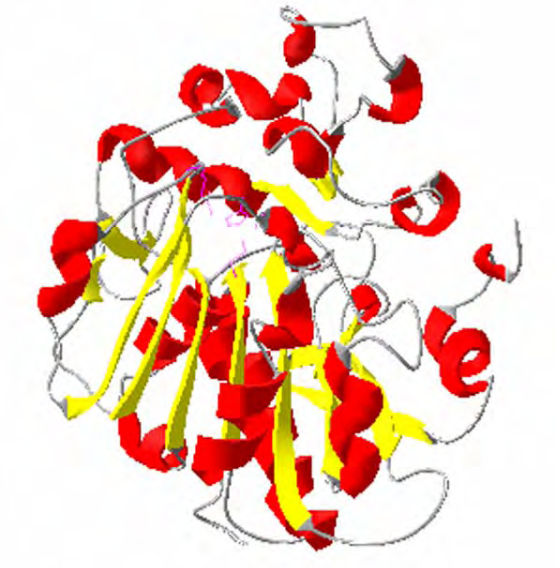

Figure 6: 3D model of the STE hydrolase Tgl1p of yeast. Beta-sheets and helical loops are shown in yellow and red. The catalytic center of the lipase can be seen in the middle of the protein with the catalytic triad residues Ser, His, and Asp coloured in purple.

2.2. Mammalian steryl ester hydrolases

2.2.1. The acid lipase gene family

The acid lipase gene family in mammals, including humans contains three acidic lipases, which are highly homologous. The three members are the human gastric lipase (HGL), the rat lingual lipase (RLL), and the human lysosomal acid lipase (HLAL or LIPA) (64). The human gastric lipase (HGL; triacylglycerol lipase; EC 3.1.1.3) is synthesized primarily by the chief cells of the fundic mucosa and secreted into the gastric lumen (65, 66). HGL is an acid stable enzyme and exhibits its main activity against triacylglycerides in the gastrointestinal tract and is required for the absorption and digestion of dietary lipids in the stomach and the duodenum (66). The molecular weight of HGL is around 43 kDa. The protein has 379 residues is highly homologous to that of the two other acidic lipases, the rat lingual lipase (RLL; 76% identity; 67) and human lysosomal acid lipase (HLAL; 58% identity; 68). Rat lingual lipase (RLL; triacylglycerol lipase; EC 3.1.1.3) consists of 377 amino acids and has a similar function as HGL. The RLL also hydrolyzes triacylglycerides in the gastrointestinal tract (66, 69, 70). Both enzymes are active within a broad pH range of 2.2 – 6.0. The human lysosomal acidic lipase (HLAL or LIPA; cholesteryl esterase; EC 3.1.1.13) is composed of 378 amino acids and has a molecular weight of 43 kDa. Unlike HGL and RLL, LIPA catalyzes the breakdown of cholesteryl esters and only to a minor extent triacylglycerides (71, 72, 73). Moreover, LIPA also exhibits its main enzymatic activity at low pH. LIPA will now be described in more detail since the main topics discussed here are sterol homeostasis and STE hydrolases.

LIPA is synthesized by virtually all nucleated cells except erythrocytes and is found primarily in lysosomes. The lipase exhibits its main activity against cholesteryl esters but is also active on triglycerides. Both, cholesteryl esters and triglycerides, are internalized via receptor mediated endocytosis of lipoprotein particles and are hydrolyzed by LIPA at an acid pH in a lysosomal compartment. The products of the enzymatic reaction are cholesterol, di- and monoglycerides, and free fatty acids. These breakdown products are then transferred via a poorly understood pathway from the lysosomes to the cytoplasm and used for energy production, biosynthetic processes, or become re-esterified again in the ER, for lipid storage. Remarkably, the liberated cholesterol also regulates endogenous cholesterol biosynthesis, uptake of LDL, and esterification of cholesterol (72). Thus LIPA also participates in the regulation of the intracellular pool of free cholesterol. Taken together, this already shows the importance of LIPA in the regulation of intracellular cholesterol homeostasis and the important function of LIPA is further emphasized by the fact that altered LIPA function has been implicated in severe pathological diseases, like the development of atherosclerosis (74).

A hallmark in atherosclerotic lesions is the aggregation of lipid-rich macrophages with excessive amounts of cholesterol and cholesteryl ester in lysosomes (58). Further development of atherosclerotic plaques requires macrophage processing of cholesteryl esters, a process in which LIPA plays a central role as the only hydrolase for cleavage of cholesteryl ester delivered to lysosomes. Another deficiency or pathology due to low activity of LIPA which results in intralysosomal storage of the lipid substrates is known as Wolman disease in infants and as more benign cholesteryl ester storage disease (CESD) in adults. The characteristics and the molecular biology of Wolman disease and CESD will be discussed in detail in the following chapter.

2.2.2. Diseases associated with mammalian steryl ester hydrolases: Wolman disease (WD) and cholesteryl ester storage disease (CESD)

Wolman disease (WD) and cholesteryl ester storage disease (CESD) are two rare autosomal recessive human diseases associated with a complete lack or greatly reduced activity of the cholesteryl ester hydrolase, LIPA (for review see 72). The deficiency of LIPA activity leads to two distinguishable pathological phenotypes, one called Wolman disease (WD) and the other cholesteryl ester storage disease (CESD). WD is a severe disease with early infantile onset and is lethal within the first years of life due to hepatosplenomegaly and adrenal calcification (89). Prominent clinical markers of WD are massive accumulation of triglycerides and cholesterol esters in the liver, adrenal gland, intestine, and in macrophages and blood vessels. CESD, on the other hand, is less severe and often remains unrecognized until adulthood, where the patients develop premature atherosclerosis (90). As in WD, CESD patients accumulate cholesteryl esters and triacylglyceroles in organs, mainly in the liver which results in hepatomegaly (91, 92). Interestingly, residual LIPA activity has been detected in CESD patients, but not in WD patients (73). Recently, mutational analysis of these residues demonstrated that Ser 153 is important for hydrolysis of triglycerides and cholesteryl esters whereas Ser 99 does not affect the enzymatic activity of LIPA (102, 104). Several mutations in LIPA have been associated with the WD and CESD, but none of them directly affects the predicted nucleophilic Ser within the lipase consensus sequence Gly-X-Ser-X-Gly. Sequence analysis of cDNA derived form mRNA of WD patients helped to identify numerous mutations in the LIPA gene. These mutations include missense mutations [L179P, (93); G321W, (93);], nonsense mutations [T22X, (95); Q277X, (96); Y303X, (97);], insertions [635insT, (93); 351insA, (98);], and deletions [159–166, (94); 435–436, (94); exon8, (99);]. The most frequent observed mutation in CESD patients is a splice donor site G to A transition

that leads to aberrant splicing of exon 8 and a 72-base pair deletion encoding amino acids 254-277 (103). It has been suggested that the primary differences in phenotype between WD and CESD is due to the residual level of LIPA activity present in various tissues. The residual activity was thought to be due to different mutations in the gene locus (92, 100, 101). However, experiments testing the enzymatic activity of heterologously expressed forms of LIPA with mutations found in CESD patients showed that no esterase activity could be detected in all mutant enzymes tested. Nevertheless, low level of triglyceride activity was still present in some cases (102). The cholesterol ester hydrolysis *in vitro* studies did not adequately reflect the *in situ* residual enzymatic activity. The key for understanding the differences between the severe and lethal WD and the milder form CESD seems to lie in compound heterozygosity in CESD chromosomes. In CESD patients at least one mutant allele has the potential to produce enough residual enzymatic function leading to a "milder" phenotype. In the majority of CESD cases this may come from a single splicing mutation in one allele, an exon 8 splice defect, which accounts for approximately 70% of mutant chromosomes in CESD patients. The CESD chromosomes thus are heterogeneous and consist of a large number of infrequent mutations including Wolman mutations which then cause CESD (103).

2.2.3. The hormone sensitive lipase HSL

Another key enzyme in mammalian lipolysis is the hormone-sensitive lipase (HSL). Probably no other mammalian lipase was subject to more intense research than the HSL. As the name implies, lipolytic activity of HSL is controlled by hormones. Of particular interest is the activation of the lipase, which occurs via reversible phosphorylation (75). HSL is phosphorylated by cAMP-dependent protein kinsae (PKA), which is stimulated by hormones, such as corticotropine and adrenaline, through the cAMP signalling cascade (76). Interestingly, phosphorylation of HSL upon liploytic stimuli not only activates but also induces relocalization the lipase to lipid droplets (77). The HSL is expressed in a variety of tissues including adipose tissue, muscle, testis, adrenal gland, and highest expression of HSL is observed in adipose tissue (78, 79). HSL displays major enzymatic activity against triacylglyceroles and diacylglycerols, which is particularly important in adipose tissue to provide free fatty acids as a main energy source (80, 81, 82). Apart from its hydrolytic activity against tri- and diacylglycerols, HSL also exhibits cholesteryl ester hydrolase activity. The activity against cholesteryl ester is thought to be important in steriodogenic tissue to provide free cholesterol for hormone production (75). Human HSL is composed of 775 amino

acids and has a molecular weight of around 84 kDa (83). Although there are no significant sequence homologies of HSL to other known lipases, it shares the same characteristic three dimensional structure, the alpha/beta hydrolase fold, of other lipases. Apart from the three dimensional structure, HSL also contains a catalytical triad consisting of Ser, Asp and His in this proper order. Like in the acidic lipases the nucleophilic active Ser of the catalytical triad is part of the lipase consensus sequence Gly-X-Ser-X-Gly (84). Although HSL activity and/or deficiency cannot be assigned to a particular human disease, there is emerging evidence that HSL functions affect and/or are involved in obesity, type-2 diabetes, and hyperlipidemia (85). Taken together, HSL is subject to intensive research since it is a key player in lipid homeostasis in mammals and studies of the reversible phosphorylation of HSL revealed deep insights into regulating mechanisms of lipases.

2.3. Yeast steryl ester hydrolases

STE hydrolase activity in yeast was first described in 1978 by Taketani *et al.*. In the beginning the enzymatic activity was localized to the mitochondrial fraction and the molecular weight of the enzyme(s) was estimated to be 70 kDa by gel filtration, although the gene(s) encoding for the enzyme(s) were not identified (86, 87). Later, Zinser *et al.* reported highest STE hydrolase activity in the plasma membrane fraction and in secretory vesicles (17). However, the genes encoding for the STE hydrolase activity still remained unknown. The first clues about the identity of STE hydrolases in yeast came from the analysis of yeast lipid particles proteins. This analysis revealed that some proteins which are found associated with lipid particles contained lipase motifs and among these a putative STE hydrolase Tgl1p was found. *TGL1* was thought to encode a STE hydrolase although its function was not clarified for a long time. Experiments showed that single deletion strains of *TGL1* exhibited elevated levels of STE (~ 40%). However, additional experiments showed that strains lacking *TGL1*, although they accumulated STE, were able to completely empty their STE pool (88). This finding already suggested that there is probably more than one STE hydrolase present in yeast. Our starting point to identify these was computational analysis of lipase-motif containing ORFs in the yeast genome which revealed 8 candidate STE hydrolases (50). Among these predicted STE hydrolases, the lipid particle protein *TGL1* and its two closest homologues *YEH1* and *YEH2* were present. Interestingly, *YEH1*, *YEH2*, and *TGL1* show highest homology to the mammalian acid lipase LIPA. Furthermore, *in vivo* and *in vitro* studies confirmed that Yeh1p, Yeh2p and Tgl1p are the STE hydrolases of yeast. A mutant strain lacking all three lipases shows no detectable STE hydrolase activity anymore, both *in*

vivo and *in vitro*. Two of the STE hydrolases, Yeh1p and Tgl1p, are associated with lipid particles whereas the third one, Yeh2p, is localized at or close to the plasma membrane (50). A detailed characterization of the three STE hydrolases will be described in a later chapter.

3. Abbreviations

ACAT, acyl-CoA-cholesterol acyltransferase; ARE1, ACAT related enzyme 1; ARE2, ACAT related enzyme 2; CESD, cholesteryl ester storage disease; ER, endoplasmatic reticulum; HGG, human gastric lipase; LDL, low density lipoprotein particle; LIPA, lysosomal acid lipase A; RLL, rat lingual lipase; STE, steryl ester; TAG, triacylglycerol; WD, Wolman disease; YEH1, steryl ester hydrolase 1; YEH2, steryl ester hydrolase 2

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CHAPTER II

YEH1, YEH2, and TGL1 encode a novel family of membrane-anchored lipases that are required for steryl ester hydrolysis in yeast

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The *Saccharomyces cerevisiae YLL012/YEH1*, *YLR020/YEH2*, and *TGL1* Genes Encode a Novel Family of Membrane-Anchored Lipases That Are Required for Steryl Ester Hydrolysis

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Sterol homeostasis in eukaryotic cells relies on the reciprocal interconversion of free sterols and steryl esters. The formation of steryl esters is well characterized, but the mechanisms that control steryl ester mobilization upon cellular demand are less well understood. We have identified a family of three lipases of *Saccharomyces cerevisiae* that are required for efficient steryl ester mobilization. These lipases, encoded by *YLL012/YEH1*, *YLR020/YEH2*, and *TGL1*, are paralogues of the mammalian acid lipase family, which is composed of the lysosomal acid lipase, the gastric lipase, and four novel as yet uncharacterized human open reading frames. Lipase triple-mutant yeast cells are completely blocked in steryl ester hydrolysis but do not affect the mobilization of triacylglycerols, indicating that the three lipases are required for steryl ester mobilization in vivo. Lipase single mutants mobilize steryl esters to various degrees, indicating partial functional redundancy of the three gene products. Lipase double-mutant cells in which the third lipase is expressed from the inducible *GAL1* promoter have greatly reduced steady-state levels of steryl esters, indicating that overexpression of any of the three lipases is sufficient for steryl ester mobilization in vivo. The three yeast enzymes constitute a novel class of membrane-anchored lipases that differ in topology and subcellular localization.

Sterols are essential lipids of eukaryotic cells. They are present in two major forms: free sterols and steryl esters. Free sterols are synthesized in the endoplasmic reticulum (ER) membrane but are greatly enriched at the plasma membrane, which harbors 90% of the free-sterol pool of a cell (27). Steryl esters, on the other hand, serve as a storage form for fatty acids and sterols that are deposited in intracellular lipid bodies or particles. The conversion of free sterols and acyl coenzymes A to steryl esters is localized to the ER (14). The formation of steryl esters is important to maintain sterol homeostasis, as the steryl ester pool conceptually serves to buffer both excess and a lack of free sterols (12). The genes required for the synthesis of steryl esters in yeast have been identified, and mutants that lack steryl esters are viable, indicating that their synthesis is not essential under standard growth conditions (52, 53). The reverse process, however, how endogenously synthesized steryl esters are mobilized from their stores and hydrolyzed to release free sterols and fatty acids, is less well understood, even though cleavage of this ester bond is generally thought to be catalyzed by a lipase.

Lipases constitute a heterogeneous family of proteins with carboxyl esterase activity and are activated by a lipid-water interface (for reviews, see references 34 and 51). Crystallographic analysis revealed that lipases are remarkably similar in structure despite low overall sequence conservation. They belong to the alpha/beta hydrolase fold family of enzymes with diverse hydrolytic functions. Their catalytic domain consist of a predominantly parallel beta-sheet structure of eight beta sheets connected by helical loops of various lengths that contain the catalytic-triad residues serine, aspartic acid, and histidine (10, 37, 42). The nucleophilic serine of this triad is itself part of the nearly ubiquitous lipase consensus sequence motif GXSXG (17). These three amino acids, assisted by the dipolar oxyanion hole, which stabilizes the charge distribution of the transition state, catalyze the hydrolysis of the ester bond (37).

Only a few gene products with lipolytic activity against neutral lipids have been characterized in *Saccharomyces cerevisiae*. Tgl1 has been proposed to be a triglyceride-specific lipase on the basis of its homology to lipases from humans and rats, but enzymatic activity of Tgl1 against triacylglycerol has never been demonstrated (1). *TGL2*, on the other hand, was found to prevent lethal accumulation of diacylglycerol (DAG) in a DAG kinase mutant *Escherichia coli* strain grown on arbutin, an artificial phosphoglycerol acceptor (49). Tgl2p displays sequence homology to *Pseudomonas* triacylglycerol lipases, and its expression in *E. coli* confers lipolytic activity against triacylglycerol and DAG with short-chain fatty acids (49). The physiological function of *TGL2* in yeast, however, has not been characterized. A third lipase, Tgl3p, is required for mobilization of triacylglycerol in vivo, and the protein confers triacylglycerol lipase activity in vitro (7).

The gene(s) encoding steryl esterase(s) in yeast has not been identified. The steryl ester-hydrolyzing activity is induced under anaerobic conditions and enriched in the membrane pellet of semianaerobically grown yeast cells (46). The activity copurified with a 70-kDa protein, but the enzyme that catalyzes the reaction has not formally been identified (45).

The aim of this study was to identify the presumed steryl ester-hydrolyzing activity in yeast. The yeast genome contains three open reading frames (ORFs) that contain an alpha/beta

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TABLE 1. *S. cerevisiae* strains used in this study

Strain	Relevant genotype	Source and/or reference
YRS1533	BY4742; *MAT*α *his3Δ1 leu2Δ0 ura3Δ0 lys2Δ0*	EUROSCARF; 50
YRS1972	*MAT*α *his3Δ1 leu2Δ0 ura3Δ0 lys2Δ0 yeh1*::*kanMX4*	EUROSCARF; 50
YRS1971	*MAT*α *his3Δ1 leu2Δ0 ura3Δ0 lys2Δ0 yeh2*::*kanMX4*	EUROSCARF; 50
YRS1973	*MAT*α *his3Δ1 leu2Δ0 ura3Δ0 lys2Δ0 tg11*::*kanMX4*	EUROSCARF; 50
YRS1948	*MAT*α *his3Δ1 leu2Δ0 ura3Δ0 lys2Δ0 yeh1*::*HIS3MX6 yeh2*::*kanMX4*	This study
YRS1837	*MAT*α *his3Δ1 leu2Δ0 ura3Δ0 lys2Δ0 yeh1*::*HIS3MX6 tg11*::*kanMX4*	This study
YRS1838	*MAT*? *his3Δ1 leu2Δ0 ura3Δ0 met15Δ0 LYS2 yeh2*::*kanMX4 tg11*::*kanMX4*	This study
YRS1840	*MAT*? *his3Δ1 leu2Δ0 ura3Δ0 met15Δ0 LYS2 yeh1*::*HIS3MX6 yeh2*::*kanMX4 tg11*::*kanMX4*	This study
YRS1974	*MAT*α *his3Δ1 leu2Δ0 ura3Δ0 lys2Δ0 YEH1-GFP-HIS3MX6*	This study
YRS1861	*MAT***a**/α *his3Δ1/his3Δ1 leu2Δ0/leu2Δ0 ura3Δ0/ura3Δ0 MET15/met15Δ0 LYS2/lys2Δ0 ERG6/ERG6-RFP-kanMX6 YEH1/YEH1-GFP-HIS3MX6*	This study
YRS2086	*MAT*α *his3Δ1 leu2Δ0 ura3Δ0 lys2Δ0 YEH2-GFP-HIS3MX6*	This study
YRS1858	*MAT***a** *his3Δ1 leu2Δ0 ura3Δ0 met15Δ0 TGL1-GFP-HIS3MX6*	This study
YRS2105	*MAT***a**/α *his3Δ1/his3Δ1 leu2Δ0/leu2Δ0 ura3Δ0/ura3Δ0 MET15/met15Δ0 LYS2/lys2Δ0 ERG6/ERG6-RFP-kanMX6 TGL1/TGL1-GFP-HIS3MX6*	This study
YRS2085	*MAT*α *his3Δ1 leu2Δ0 ura3Δ0 lys2Δ HIS3MX6-GAL1-GFP-YEH1*	This study
YRS2083	*MAT*α *his3Δ1 leu2Δ0 ura3Δ0 lys2Δ HIS3MX6-GAL1-GFP-YEH2*	This study
YRS2084	*MAT*α *his3Δ1 leu2Δ0 ura3Δ0 lys2Δ HIS3MX6-GAL1-GFP-TGL1*	This study
YRS1953	*MAT***a** *his3Δ1 leu2Δ0 ura3Δ0 met15Δ0 LYS trp1Δ*	This study
YRS1956	*MAT*? *his3Δ1 leu2Δ0 ura3Δ0 met15Δ0 LYS2 trp1Δ yeh1*::*HIS3MX6 yeh2*::*kanMX4tgl1*::*kanMX4*	This study
YRS2090	*MAT*? *his3Δ1 leu2Δ0 ura3Δ0 met15Δ0 LYS2 trp1Δ yeh2*::*kanMX4 tgl1*::*kanMX4 YEH1-GFP-TRP1*	This study
YRS2091	*MAT*α *his3Δ1 leu2Δ0 ura3Δ0 lys2Δ0 trp1Δ yeh1*::*HIS3MX6 tgl1*::*kanMX4 YEH2-GFP-TRP1*	This study
YRS2092	*MAT*α *his3Δ1 leu2Δ0 ura3Δ0 lys2Δ0 trp1Δ yeh1*::*HIS3MX6 yeh2*::*kanMX4 TGL1-GFP-TRP1*	This study
YRS2161	*MAT*? *his3Δ1 leu2Δ0 ura3Δ0 met15Δ0 LYS2 trp1Δ yeh2*::*kanMX4 tgl1*::*kanMX4 TRP1-PGAL1-GFP-YEH1*	This study
YRS2162	*MAT*α *his3Δ1 leu2Δ0 ura3Δ0 lys2Δ0 trp1Δ yeh1*::*HIS3MX6 tgl1*::*kanMX4 TRP1-PGAL1-GFP-YEH2*	This study
YRS2163	*MAT*α *his3Δ1 leu2Δ0 ura3Δ0 lys2Δ0 trp1Δ yeh1*::*HIS3MX6 yeh2*::*kanMX4 TRP1-PGAL1-GFP-TGL1*	This study

hydrolase-associated lipase region, as classified by the pfam database of protein families. To determine whether one or more of these genes is required for steryl ester hydrolysis, we used an in vivo assay to monitor the mobilization of radiolabeled steryl ester in mutant strains lacking one, two, or all three of these candidate lipases. The results of this analysis indicate that together these three lipases account for all of the steryl ester-hydrolyzing activity that is present in yeast. Unexpectedly, all three yeast lipases appear to be membrane anchored, which makes them the first, to our knowledge, membrane-anchored neutral lipid lipases to be described. Their subcellular distribution and possible membrane topology with respect to the localization of the active site are discussed.

MATERIALS AND METHODS

Yeast strains and growth conditions. The yeast strains used in this study are listed in Table 1. Strains bearing single deletions of nonessential genes were obtained from EUROSCARF (www.rz.uni-frankfurt.de/FB/fb16/mikro/euroscarf/index.html) (50). Strains were cultivated in YPD rich medium (1% Bacto Yeast Extract, 2% Bacto Peptone [U.S. Biological, Swampscott, Mass.], 2% glucose) or in minimal medium. Selection for the *kanMX4* marker was on medium containing G418 (Gibco BRL, Life Technologies) at 200 μg/ml. Double- and triple-mutant strains were generated by crossing of single mutants and by gene disruption with the PCR deletion cassettes (31) and the primers listed in Table 2. Wild-type and double- and triple-mutant strains were converted to tryptophan auxotrophy with plasmid pNKY1009 (*trp1::URA3*) and converted back to uracil auxotrophy by selection on FOA medium (2). Yeast was transformed by lithium acetate (25).

Green fluorescent protein (GFP) tagging, subcellular fractionation, and Western blot analysis. N- and C-terminal GFP tagging of *YEH1*, *YEH2*, and *TGL1* was performed by homologous recombination with the PCR fusion cassette from pFA6a-HIS3MX6-PGAL1-GFP(S65T), pFA6a-TRP1-PGAL1-GFP(S65T), pFA6a-GFP(S65T)-TRP1, and pFA6a-GFP(S65T)-HIS3MX6 (31) and the primers listed in Table 2. The resulting PCR fragments were transformed into wild-type strain BY4742 or BY4741, and correct integration of the fusion cassette was confirmed by colony PCR with the control primers listed in Table 2.

Protein concentrations were determined by the method of Lowry et al. (33), with the Folin reagent and bovine serum albumin as the standard. Proteins were precipitated with 10% trichloroacetic acid (TCA), resuspended in sample buffer, and separated by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis. Western blots were probed with rabbit antisera against GFP (1:5,000; Torrey Pines Biolabs, Inc., Houston, Tex.), Kar2 (1:5,000; M. Rose, Princeton University), Erg6 and porin (both 1:10,000; kind gifts from G. Daum, Graz University

TABLE 2. Primers used in this study

Primer name	Sequence (5′–3′)[a]	Purpose
YEH1; fwdYLLko	TTTTAATATAGATGGCTGCAGATAAAGTAATAGTTTTATAcggatccccgggttaattaa	Forward primer for *YEH1*::*HIS3MX6* cassette
YEH1; revYLLko	AAATGTATCGTTATGCACGGTGATGTCCCAACTCCCGACAgaattcgagctcgtttaaac	Reverse primer for *YEH1*::*HIS3MX6* cassette
YEH1; upYLLreg	ACGTATCGCAGGCAATGTGC	Forward control primer for gene deletion
YEH1; F2yllGFP	ACAGAGGTGGAAACGGAGCTGGAAATGGTTGCTGAGAAGcggatccccgggttaattaa	Forward primer for C-terminal GFP tagging of *YEH1*
YEH1; R1yllGFP	AAATGTATCGTTATGCACGGTGATGTCCCAACTCCCGACAgaattcgagctcgtttaaac	Reverse primer for C-terminal GFP tagging of *YEH1*
YEH1; regYLLfwd	ACGCATCAAAGGCCCCAAAT	Forward control primer for C-terminally tagged *YEH1*
YEH1; F4Yehprom	AATACTTATTTTTTAATATAGATGGCTGCAGATAAAGTAAgaattcgagctcgtttaaac	Forward primer for N-terminal PGAL1-GFP tag
YEH1; R5yehgfpN	TAAATTCCTAGCTCTTTTCAACACCGCAGAAACACCTGCtttgtatagttcatccatgc	Reverse primer for N-terminal PGAL1-GFP tag
YEH1; revYEHreg	TTGTCATGTTCCAGATCGAGG	Reverse control primer for N-terminal PGAL1-GFP tag
YEH2; F2ylrGFP	ATAGATAGAATTGGTAAGCCAATGATAGAAAATTTGAGGcggatccccgggttaattaa	Forward primer for C-terminal GFP tagging of *YEH2*
YEH2; R1ylrGFP	TTTACGTGTAACATCTACAGATACATATATCCGTATATACcaattcgagctcgtttaaac	Reverse primer for C-terminal GFP tagging of *YEH2*
YEH2; regYLRmid	GTGAGGCTAATGGCAAAAGG	Control primer for C-terminal tag
YEH2; F4YLRprom	TTTCTCATCAAAGGTATAATTAAAATTGTTGGGAAATACTgaattcgagctcgtttaaac	Forward primer for N-terminal PGAL1-GFP tag
YEH2; R5YLRgfpN	CACCAACCGCTGAACCTCATCAACCACCTTATTTACTGCtttgtatagttcatccatgc	Reverse primer for N-terminal PGAL1-GFP tag
YEH2; revYLRreg	GGGCGTTGTGGAAGATATTGA	Reverse control primer for N-terminal PGAL1-GFP tag
YEH2; regYLR450up	CCACCCGTGTTTCCTAGGTT	Forward control primer for gene deletion
TGL1; F2tglGFP	CGACAACTAGATGCCAACTCTTCGACAACTGCGCTGGATcggatccccgggttaattaa	Forward primer for C-terminal GFP tagging of *TGL1*
TGL1; R1tglGFP	GATATTAAGACTTCTTATGAAATCCATTTATTGTGTATAgaattcgagctcgtttaaac	Reverse primer for C-terminal GFP tagging of *TGL1*
TGL1; regTGL250up	TCCCGGATACTTATTGAAGCTA	Forward control primer for C-terminal GFP tag and for gene deletion
TGL1; F4TGLprom	AACAAGGAAAGAAGAAAGAAAACAATTCGAACAAAACTTTgaattcgagctcgtttaaac	Forward primer for N-terminal PGAL1-GFP tag
TGL1; R5TGLgfpN	ATCTGTCGATAATCTGCCTAAAAAGGGGAAGTATGCtttgtatagttcatccatgc	Reverse primer for N-terminal PGAL1-GFP tag
TGL1; revTGLreg	TACCAAATGATCCTCGACAG	Reverse control primer for N-terminal PGAL1-GFP tag
HIS5; His5contrr	TACGGGCGACAGTCACATC	Reverse control primer for *HIS3MX6*
GFP; revGFPkontr	TTCGGGCATGGCACTCTTGA	Reverse control primer for GFP

[a] Sequences in uppercase indicate homology to the genome of *S. cerevisiae*, and those in lowercase indicate homology to the PCR cassette used.

of Technology), Sed5p (1:3,000; a kind gift from H. Pelham, MRC Cambridge), Tlg1p (1:1,000; a kind gift from H. Pelham), Mnn1p (1:400, a kind gift from T. Graham, Vanderbilt University, Nashville, Tenn.), Pma1 (1:50,000), Gas1 (1:5,000, a kind gift from A. Conzelmann, University of Fribourg), or mouse anti-Pho8p (2 μg/ml; Molecular Probes).

Fractionation on an Accudenz density gradient was performed essentially as described by Cowles et al. (16). One hundred optical density (OD) units of exponentially growing cells were collected by centrifugation (3,000 × *g*, 10 min), resuspended in 100 mM Tris (pH 9.4)–10 mM dithiothreitol, and incubated for 10 min at 30°C. Cells were pelleted (3,000 × *g*, 5 min) and resuspended in 0.2× YPD medium–0.6 M sorbitol–10 mM KP_i, pH 7.5. Cells were converted to spheroplasts by incubation with 16 U of Zymolyase (Seikagaku Corporation, Tokyo, Japan) for 20 min at 30°C. Spheroplasts were pelleted (4,000 × *g*, 20 min), resuspended in lysis buffer A (0.2 M sorbitol–50 mM potassium acetate–20

mM HEPES [pH 6.8]–2 mM EDTA–0.2 mM phenylmethylsulfonyl fluoride [PMSF] supplemented with a protease inhibitor cocktail [Complete; Roche-Diagnostics, Rotkreuz, Switzerland]), and lysed with a Dounce homogenizer. The homogenate was layered on top of an 8 to 43% Accudenz gradient (Accurate Chemical and Scientific Corp.) prepared in nine steps in lysis buffer A as described by Cowles et al. (16). The gradient was then centrifuged in a swing-out rotor (Sorvall TH641; Kendro Laboratory Products, Asheville, N.C.) at 170,000 $\times g$ for 18 h at 4°C. Eleven fractions of equal volume were collected from the top of the gradient, proteins were precipitated, and the distribution of marker proteins was analyzed by Western blotting.

To determine the membrane association of the GFP-tagged lipases, exponentially growing cells were pelleted by centrifugation (3,000 $\times g$, 10 min), resuspended in lysis buffer A, converted to spheroplasts, and lysed as described above. The homogenate was precleared by centrifugation at 500 $\times g$ for 10 min and then separated into pellet (P13) and supernatant (S13) fractions by centrifugation at 13,000 $\times g$ for 20 min. The S13 fraction was then further separated into a 30,000 $\times g$ pellet (P30) and supernatant (S30) by centrifugation at 30,000 $\times g$ for 30 min. The S30 was fractionated again by centrifugation at 100,000 $\times g$ for 30 min to obtain a 100,000 $\times g$ pellet (P100) and supernatant (cytosol). All centrifugation steps were performed at 4°C.

To determine the membrane association and topology of the GFP-tagged lipases, detergent and salt extractions and proteinase K protection experiments were performed essentially as previously described (47). Detergent and salt extractions of microsomal membranes (P13) were performed by incubating 50 μg of the microsomal fraction with either 1% Triton X-100, 7% SDS, 1 M NaCl, or 0.1 M Na_2CO_3 in lysis buffer A without protease inhibitors for 30 min on ice. Samples were then centrifuged at 13,000 $\times g$ for 30 min, and proteins from the pellet and supernatant fractions were analyzed by Western blotting. For the proteinase K protection experiment, microsomes (50 μg) were incubated with 9, 14, or 28 μg of proteinase K per ml for 30 min on ice. The reaction was stopped by addition of PMSF (5 mM), and proteins were precipitated with 10% TCA. The pellet was dissolved in sample buffer, heated to 95°C for 5 min, and subjected to SDS-polyacrylamide gel electrophoresis and Western blot analysis.

Vacuoles and lipid particles were isolated by flotation as previously described (29). Briefly, spheroplasts were resuspended in lysis buffer B (12% Ficoll, 10 mM MES-Tris [pH 6.9], 0.2 mM EDTA) and lysed with a Dounce homogenizer. The lysate was cleared by centrifugation at 5,000 $\times g$ for 5 min. The supernatant was placed at the bottom of an ultracentrifuge tube and overlaid with lysis buffer. Lipid particles were subjected to flotation by centrifugation at 100,000 $\times g$ for 1 h. The floating fraction was removed, diluted with lysis buffer, and placed at the bottom of a second ultracentrifuge tube. The sample was overlaid with 8% Ficoll–10 mM MES-Tris (pH 6.9)–0.2 mM EDTA and centrifuged again at 100,000 $\times g$ for 1 h. The second flotation product was removed and diluted with 0.6 M sorbitol–8% Ficoll–10 mM MES-Tris (pH 6.9)–0.2 mM EDTA. The sample was then placed at the bottom of a third ultracentrifuge tube filled with 0.25 M sorbitol–10 mM MES-Tris (pH 6.9)–0.2 mM EDTA and again subjected to flotation by centrifugation at 100,000 $\times g$ for 1 h. The third flotation product was greatly enriched in lipid particle markers, whereas the pellet was enriched in vacuolar marker proteins.

Plasma membrane was enriched by centrifugation through a sucrose step gradient as previously described (43). Cells were harvested, resuspended in lysis buffer D (20 mM Tris [pH 8.5], 5 mM EDTA, 1 mM PMSF), and broken by vigorous agitation with 0.3-mm-diameter glass beads with a Merkenschlager homogenizer (B. Braun Biotech, Melsungen, Germany) equipped with CO_2 cooling. The lysate was cleared by centrifugation (5,000 $\times g$, 10 min), and membranes were pelleted by centrifugation (20,000 $\times g$ 10 min). Membranes were resuspended in TEDG (10 mM Tris HCl [pH 7.5], 1 mM EDTA, 20% glycerol, 0.2 mM dithiothreitol) and layered on top of a sucrose step gradient (53 to 43%, prepared in TEDG). This gradient was centrifuged at 120,000 $\times g$ for 2 h, and the membranes from the interface were collected with a syringe. The plasma membrane fraction was diluted in 10 mM Tris–1 mM EDTA and finally pelleted by centrifugation (20,000 $\times g$, 20 min).

Fluorescence microscopy. In vivo localization of GFP-tagged versions of Yeh1, Yeh2, and Tgl1 was performed by fluorescence microscopy with a Zeiss Axioplan 2 (Carl Zeiss, Oberkochen, Germany) equipped with an AxioCam charge-coupled device camera and AxioVision 3.1 software. Lipid droplets were visualized with red fluorescent protein (RFP)-tagged Erg6 as a marker protein (24), and nuclei were visualized by 4′,6′-diamidino-2-phenylindole (DAPI) staining.

In vivo neutral lipid mobilization. The neutral lipid pool was labeled by incubating the cells with 10 μCi of [^{3}H]palmitic acid (American Radiolabeled Chemicals Inc., St. Louis, Mo.) per ml for 16 h at 24°C. Cells were then washed and diluted in liquid YPD medium containing either 10 μg of cerulenin (ICN Biomedicals, Irvine, Calif.) per ml or 30 μg of terbinafine (a kind gift from N. Ryder [Novartis Research Institute, Vienna, Austria]) per ml. Aliquots of cells were removed at the time points indicated. Cells were frozen and broken with glass beads, and lipids were extracted with chloroform-methanol (1:1, vol/vol). Radioactivity in the lipid extract was determined by scintillation counting, and equal counts were brought to dryness. Lipids were separated on thin-layer chromatography (TLC) plates (Merck, Darmstadt, Germany) developed in petroleum ether-diethyl ether-acetic acid (70:30:2, vol/vol) and quantified by scanning with a Berthold Tracemaster 40 Automatic TLC-Linear Analyzer. TLC plates were then exposed to a tritium-sensitive screen and visualized with a phosphorimager (Bio-Rad Laboratories, Hercules, Calif.).

Overexpression and quantification of the endogenous steryl ester pool. The strains indicated were cultivated in rich medium containing either glucose (repressing conditions) or galactose (inducing conditions) as a carbon source. The neutral lipid pool was labeled to steady-state levels by incubating the cells with 10 μCi of [^{3}H]palmitic acid (American Radiolabeled Chemicals Inc.) per ml for 16 h at 24°C. Cells were diluted in YPD or YPGal (1% Bacto Yeast Extract, 2% Bacto Peptone [U.S. Biological], 2% galactose) liquid medium containing terbinafine (30 μg/ml). Aliquots of cells were removed at the time points indicated, and 3 OD units were used for Western blot analysis. The remaining cells (~20 OD units) were washed and broken, and lipids were extracted as described above. Equal counts were brought to dryness, and lipids were separated on TLC plates, developed in petroleum ether-diethyl ether-acetic acid (70:30:2, vol/vol), and quantified by scanning with a Berthold Tracemaster 40 Automatic TLC-Linear Analyzer.

In vitro assay for steryl ester hydrolase activity. Overnight cultures of strains were broken with glass beads, and the membrane pellet was solubilized with 1% Triton X-100 in 50 mM Tris (pH 7.0)–10% glycerol–1 mM $MnCl_2$ for 60 min at 4°C. Steryl ester hydrolase activity was determined with 100 to 300 μg of solubilized protein and cholesterol–[1-^{14}C]oleate (specific activity, 50 to 60 mCi/mmol; American Radiolabeled Chemicals Inc.) as the substrate as previously described (46). The assay was performed at 30°C for 60 min in a reaction mixture containing 100 mM phosphate buffer (pH 6.9)–0.3% Triton X-100–26 nmol substrate. The reaction was stopped by addition of chloroform-methanol (1:2; vol/vol), lipids were extracted and separated by TLC with petroleum ether-diethyl ether-acetic acid (70:30:2, vol/vol) as a solvent system, and liberated fatty acids were quantified by scanning with a Berthold Tracemaster 40 Automatic TLC-Linear Analyzer. TLC plates were then visualized with a phosphorimager (Bio-Rad Laboratories).

RESULTS

***YLL012/YEH1*, *YLR020/YEH2*, and *TGL1* are required for efficient in vivo mobilization of steryl esters.** To identify the gene(s) required for steryl ester hydrolysis in yeast, we undertook a candidate gene approach and examined the rate of in vivo mobilization of steryl esters in mutants lacking putative lipase-encoding genes. Strains harboring deletions of the genes of interest were labeled for 16 h in the presence of [^{3}H]palmitate and mobilization of storage lipids was monitored after diluting cells in medium containing terbinafine, an inhibitor of sterol biosynthesis (26). Samples were withdrawn after different time points, lipids were extracted, and the relative content of steryl esters and triacylglycerols was analyzed after TLC separation of neutral lipids. This analysis revealed a reduced rate of steryl ester mobilization in cells lacking *YLL012*, hereafter referred to as *YEH1* (yeast steryl ester hydrolase 1). Yeh1 belongs to a family of three yeast ORFs that contain an alpha/beta hydrolase-associated lipase region as classified by the pfam database of protein families (release 12.0; http://pfam.wustl.edu). Lack of the other two candidate ORFs bearing this hydrolase-associated lipase region, *YLR020/YEH2* and *TGL1*, on the other hand, did not significantly affect the rate of steryl ester mobilization under these assay conditions (Fig. 1).

Steryl ester hydrolysis is reduced in *yeh1*Δ mutant cells but not completely abolished, indicating the presence of additional, redundant, activities. To test whether *YEH2* and/or *TGL1* contribute to the residual steryl ester-hydrolyzing activ-

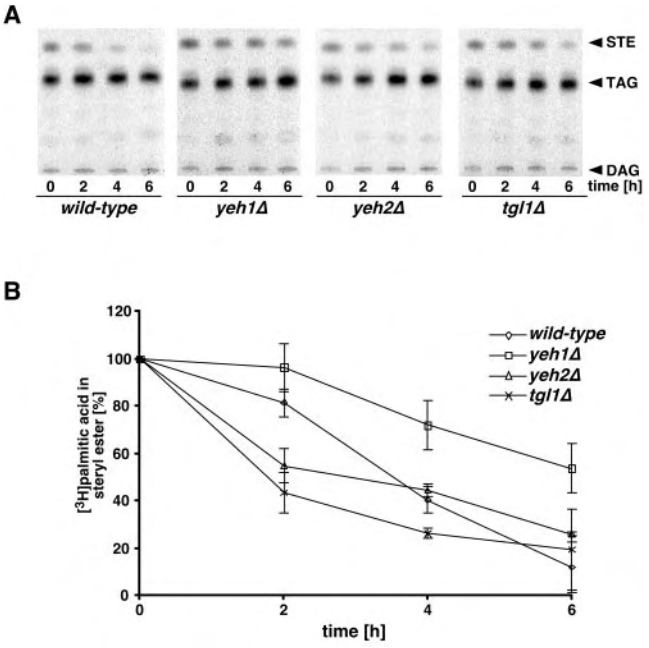


FIG. 1. Lack of *YEH1* affects the efficiency of steryl ester mobilization in vivo. (A) Wild-type (BY4742, YRS1533) and *yeh1*Δ (YRS1972), *yeh2*Δ (YRS1971), and *tgl1*Δ (YRS1973) mutant cells were labeled for 16 h with [^{3}H]palmitic acid, and the kinetics of steryl ester mobilization in vivo was analyzed by determining steryl ester levels at 0, 2, 4, and 6 h after dilution of the cells in medium containing terbinafine. Lipids were extracted and analyzed by TLC as described in Materials and Methods. TAG, triacylglycerol; STE, steryl esters. (B) The content of [^{3}H]palmitic acid-labeled steryl ester was quantified by radioscanning of TLC plates and set in relation to the levels at time zero (100%). Values represent means and standard deviations of two independent experiments.

ity observed in *yeh1*Δ mutant cells, double- and triple-mutant strains were generated and tested for steryl ester hydrolysis in vivo. This analysis revealed that steryl ester hydrolysis is substantially reduced in any one of the three double mutants and completely blocked in a *yeh1*Δ *yeh2*Δ *tgl1*Δ triple mutant (Fig. 2). Interestingly, steryl ester levels steadily increased in the triple mutant, indicating that steryl ester synthesis is ongoing while their mobilization is completely blocked. These results indicate that all of the members of this lipase family contribute to steryl ester hydrolysis in vivo and that the lack of all three lipases cannot be compensated for by other activities. The observation that the triple-mutant strain grows like wild-type cells furthermore indicates that steryl ester hydrolysis is a nonessential process under standard growth conditions (data not shown).

Lack of *YEH1*, *YEH2*, and *TGL1* does not affect triacylglycerol mobilization. To examine the apparent substrate specificity of the three lipases in vivo, single-, double-, and triple-mutant cells were labeled with [^{3}H]palmitate, but this time neutral lipid mobilization was induced by inhibiting de novo fatty acid synthesis with cerulenin (38). Under these assay conditions, wild-type cells efficiently mobilized triacylglycerols over a 3-h time period (Fig. 3). The lipase mutants, on the other hand, did not affect triacylglycerol mobilization, indicating that this family of lipases is important for efficient in vivo mobilization of steryl esters rather than triacylglycerols.

***YEH1*, *YEH2*, and *TGL1* are paralogues of the human acid lipase family.** Sequence analysis of Yeh1, Yeh2, and Tgl1 revealed that they are paralogues of the human acid lipase family, which contains the lysosomal acid lipase A, LIPA/LAL, and the secreted gastric lipase LIPF (5; for a review, see reference 34). Further inspection of the human genome sequence revealed the presence of four additional members of this lipase family, LIPL1 to LIPL4, that together with LIPF and LIPA form a lipase gene cluster on chromosome 10q23 (http://www.ensembl.org/). Multiple-sequence alignment of the three yeast steryl ester hydrolases with the two human acid lipases, LIPA and LIPF, and two of the predicted lipases, LIPL1 and LIPL3, reveals conservation of the catalytic-triad residues (Fig. 4). LIPL2 and LIPL4 were not included in this alignment as their coding sequences have not been completely predicted.

The alignment further reveals complete conservation of the GXSXG lipase motif in five of the seven lipases. In Yeh1, the first glycine of this lipase motif is replaced by a serine and in Yeh2 it is replaced by an alanine. The functionally important cysteine residues of LIPA (Cys248 and Cys257), however, are conserved in Yeh1 only (30, 40). The predicted molecular masses of these lipases range from 62 to 66 kDa, which are in good agreement with the experimentally determined 70-kDa molecular mass of the enriched yeast steryl ester hydrolase activity (45). Interestingly, all three yeast proteins are predicted to be integral membrane proteins with one to three

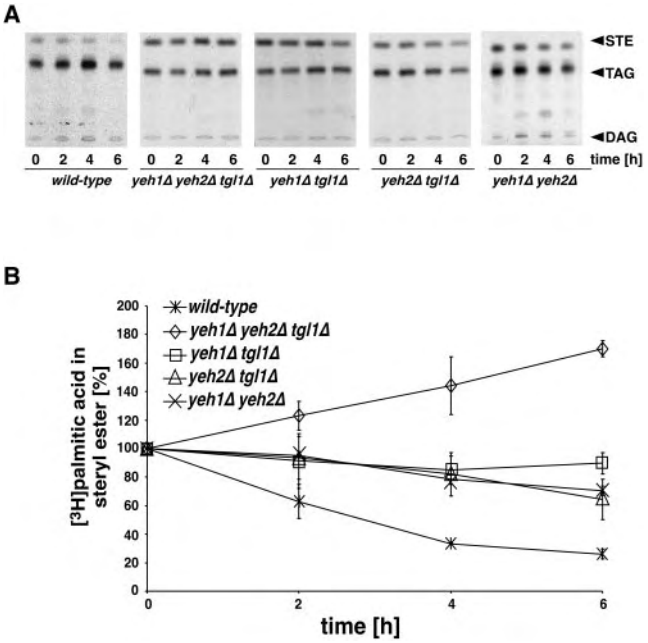


FIG. 2. *yeh1*Δ *yeh2*Δ *tgl1*Δ triple-mutant cells fail to mobilize steryl esters in vivo. (A) Lipase triple-mutant (*yeh1*Δ *yeh2*Δ *tgl1*Δ, YRS1840) and double-mutant (*yeh1*Δ *tgl1*Δ, YRS1837; *yeh2*Δ *tgl1*Δ, YRS1838; *yeh1*Δ *yeh2*Δ, YRS1948) cells were labeled for 16 h with [^{3}H]palmitic acid, and the kinetics of steryl ester mobilization in vivo was analyzed after dilution of the cells in medium containing terbinafine. Lipids were extracted and analyzed by TLC as described in Materials and Methods. TAG, triacylglycerol; STE, steryl esters. (B) The content of [^{3}H]palmitic acid-labeled steryl ester was quantified by radioscanning of TLC plates. Values represent means and standard deviations of two independent experiments.

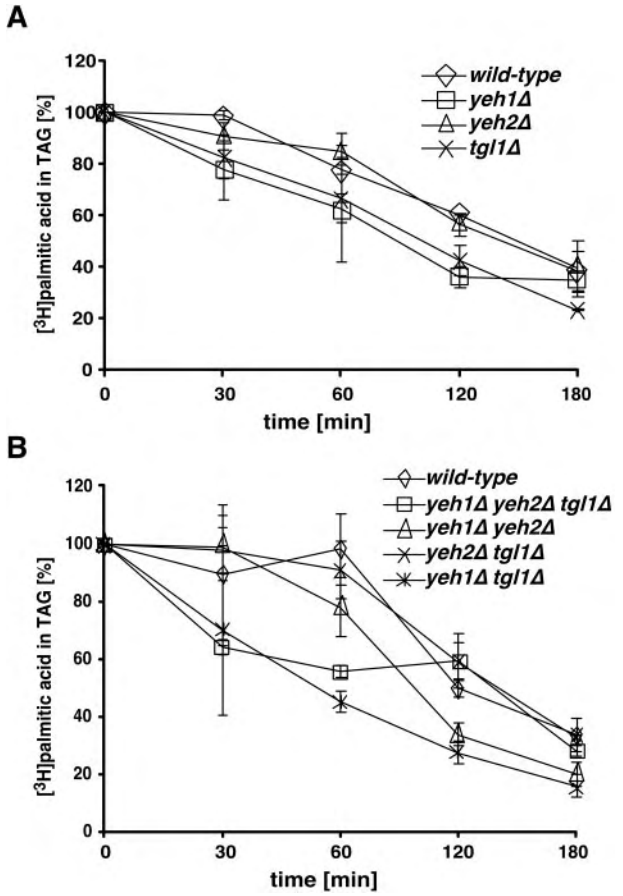


FIG. 3. *YEH1*, *YEH2*, and *TGL1* are not required for efficient mobilization of triacylglycerol. (A) Wild-type (BY4742, YRS1533) and *yeh1*Δ (YRS1972), *yeh2*Δ (YRS1971), and *tgl1*Δ (YRS1973) mutant cells were labeled for 16 h with [^{3}H]palmitic acid, and the kinetics of triacylglycerol (TAG) mobilization was analyzed after dilution of the cells in medium containing cerulenin. Lipids were extracted and analyzed by TLC, and the relative content of [^{3}H]palmitic acid-labeled TAG was quantified by radioscanning of TLC plates. Values represent means and standard deviations of two independent experiments. (B) Wild-type (BY4742, YRS1533) and lipase triple-mutant (*yeh1*Δ *yeh2*Δ *tgl1*Δ, YRS1840), and double-mutant (*yeh1*Δ *tgl1*Δ, YRS1837; *yeh2*Δ *tgl1*Δ, YRS1838; *yeh1*Δ *yeh2*Δ, YRS1948) cells were labeled for 16 h with [^{3}H]palmitic acid, and the kinetics of TAG mobilization was analyzed as described for panel A.

putative transmembrane domains and one to three potential N-glycosylation sites. The number and position of the predicted membrane domains, however, varied considerably, depending on the type of algorithm used (Table 3). The fact that the results of these predictors vary considerably may be explained in part by the fact that these proteins accommodate hydrophobic substrates into a cleft of the protein that may be mistaken as a potential transmembrane domain.

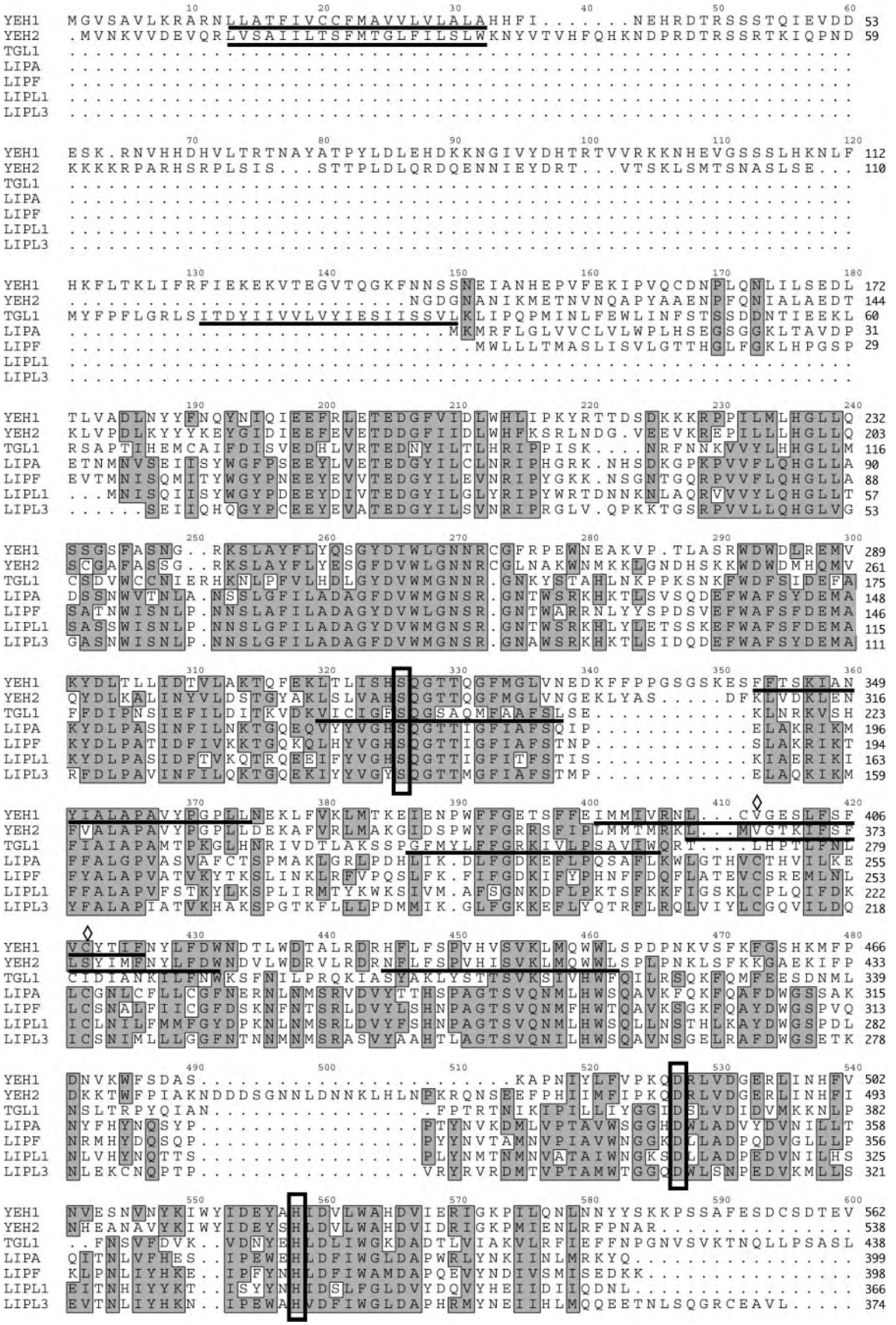
Subcellular localization of the three yeast lipases. To determine the subcellular location of the three lipases, their genomic copies were tagged to produce fusion proteins with C-terminally appended GFP by homologous recombination. All fusions were functional, as revealed by their in vivo activity in a double-mutant background (Fig. 5A). Fluorescence microscopy of strains expressing Yeh1-GFP and Tgl1-GFP revealed staining of punctate intracellular structures that colocalized with RFP-tagged Erg6, a marker protein for lipid particles (8, 24) (Fig. 5B). The C-terminally tagged copy of Yeh2 failed to give a fluorescence signal even though the fusion protein was expressed abundantly, as determined by Western blotting with anti-GFP antibody (Fig. 5C). Lack of fluorescence of the Yeh2-GFP fusion might indicate that the GFP domain of the fusion protein may not fold correctly to form the chromophore or that the half-life of the protein may be too short for its formation (22). The subcellular localization of Yeh2 was thus determined by using an N-terminally GFP-tagged version of Yeh2, driven by a galactose-inducible *GAL1* promoter. To localize GFP-Yeh2, cells were first grown in raffinose-containing medium and then shifted to galactose medium for 4 h to induce expression of the reporter. Examination of these cells revealed punctate staining of the cell periphery, suggestive of a possible plasma membrane localization of GFP-Yeh2 (Fig. 5B).

To determine whether these three lipases are indeed membrane associated, as predicted on the basis of their sequences, their fractionation properties were examined by differential centrifugation. All three proteins were slightly enriched (about threefold) in the 13,000 $\times$ *g* membrane pellet but were not detectable in the cytosol, consistent with the membrane association of all three lipases (Fig. 5C).

To confirm the localization of Yeh1-GFP to lipid particles, lipid particles and associated vacuoles were isolated by subcellular fractionation and the degree of enrichment of Yeh1-GFP in these fractions was determined by Western blot analysis. Consistent with its microscopic localization, Yeh1-GFP is ~11-fold enriched in the lipid particle fraction but hardly detectable in the vacuolar fraction (Fig. 5D). The second anti-GFP-reactive band, which migrates faster than the major ~93-kDa band, is likely due to N-terminal degradation of Yeh1-GFP. This lipid particle fraction is highly enriched in its marker protein, Erg6 (~350-fold). We did not attempt to localize Tgl1 by fractionation because it is known to be a major lipid particle protein (8).

Since C-terminally GFP-tagged Yeh2 could not be localized by microscopy, its fractionation properties on an Accudenz density gradient were examined. Western blot analysis of Yeh2-GFP yields two major bands of ~91 and ~81 kDa, which appear to be due to some as yet unidentified modification of the protein. These two major and probably mature forms of Yeh2-GFP (Fig. 5C) were enriched in fractions 2 and 3 of the Ac-

FIG. 4. Sequence alignment of the three yeast lipases with the mammalian acid lipase family. Sequence alignment of yeast lipases Yeh1 (Q07804), Yeh2 (Q07950), and Tgl1 (P34163) with mammalian acid lipase family members LIPA (P38571), LIPF (P07098), LIPL1 (Q5W064), and LIPL3 (Q5VYY2). Residues of the catalytic triad are boxed. The two functionally important cysteine residues of LIPA are indicated by open diamonds. The positions of potential transmembrane domains in the three yeast lipases, as predicted by TMpred (http://www.ch.embnet.org/software/TMPRED_form.html) and Phobius (http://phobius.cgb.ki.se/), are indicated by horizontal lines. Alignment was performed with ClustalW (http://www.ebi.ac.uk/clustalw/), and regions of homology were drawn by JavaShade (http://industry.ebi.ac.uk/JavaShade/).



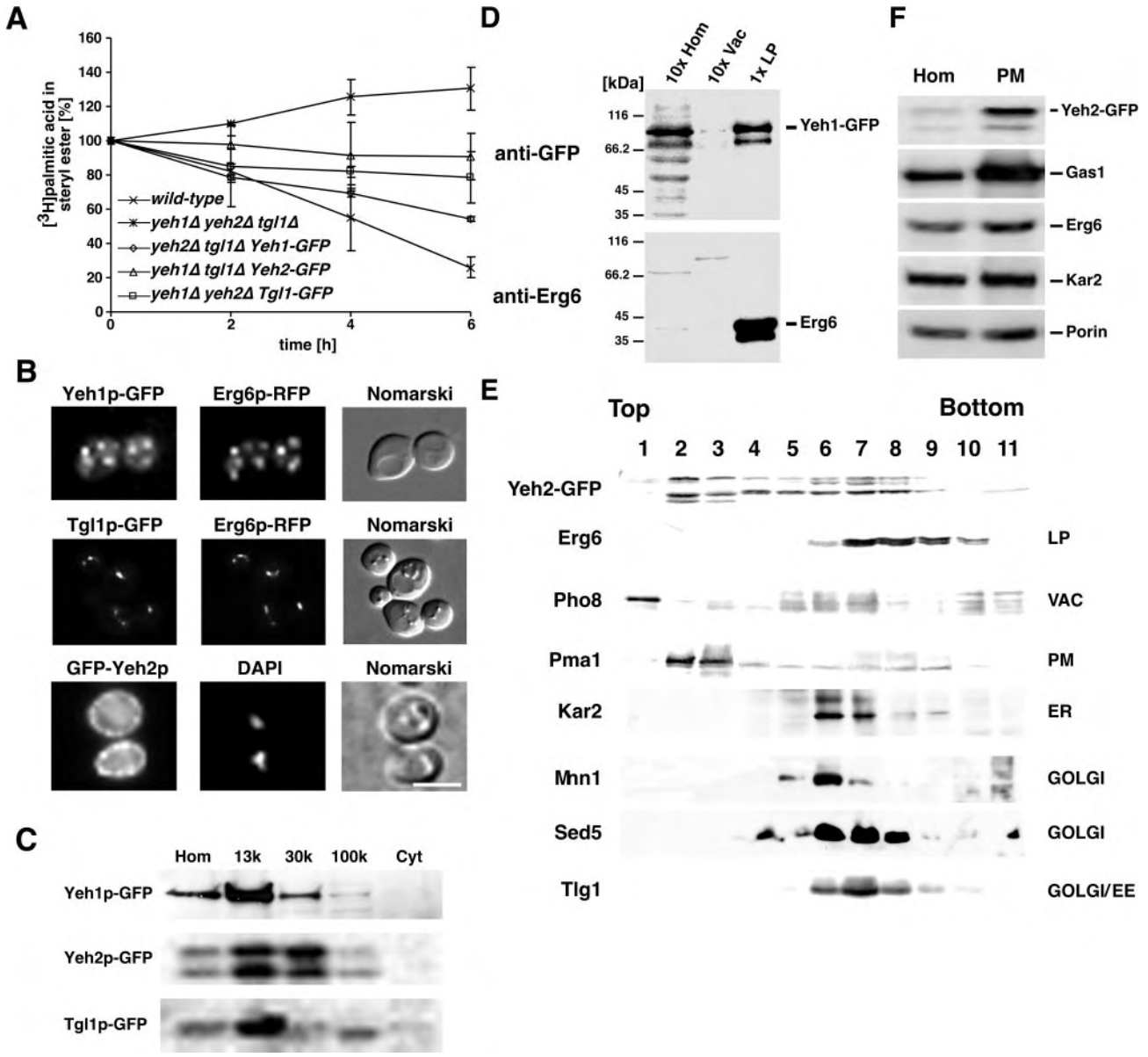


FIG. 5. Subcellular localization of Yeh1, Yeh2, and Tgl1. (A) Functionality of the GFP-tagged lipases. Wild-type (YRS1953), triple-mutant (YRS1956), and double-mutant cells in which the remaining lipase is C-terminally tagged with GFP (YRS2090, YRS2091, and YRS2092) were labeled for 16 h with [^{3}H]palmitic acid, and the kinetics of steryl ester mobilization in vivo was analyzed by determining steryl ester levels at 0, 2, 4, and 6 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-labeled steryl ester was quantified by radioscanning of TLC plates and set in relation to the levels at time zero (100%). Values represent means and standard deviations of two independent experiments. (B) In vivo localization of Yeh1-GFP, GFP-Yeh2, and Tgl1-GFP. Heterozygous diploid cells expressing chromosomally tagged versions of Yeh1-GFP and Erg6-RFP (YRS1861) or Tgl1-GFP and Erg6-RFP (YRS2105) were grown in YPD medium and examined by fluorescence microscopy. N-terminally GFP-tagged Yeh2 (YRS2085) was localized after induction in galactose-containing medium for 4 h and stained for DNA with DAPI. Bar, 5 μm. (C) Yeh1, Yeh2, and Tgl1 are membrane associated. Homogenates (Hom) from cells expressing the GFP-tagged lipases were fractionated by differential centrifugation to yield 13,000 × *g* (13k), 30,000 × *g* (30k), and 100,000 × *g* (100k) membrane pellets and cytosolic supernatants (Cyt). Ten-microgram samples of proteins from each fraction were separated by electrophoresis, blotted, and probed with an anti-GFP antibody. (D) Yeh1 is enriched in lipid particles. Haploid cells expressing Yeh1-GFP (YRS1974) were cultivated in rich medium. Vacuoles and lipid particles were isolated by flotation. Homogenate (Hom, 10 μg), vacuole (Vac, 10 μg), and lipid particle (LP, 1 μg) proteins were separated by electrophoresis, blotted, and probed with antibodies against GFP (anti-GFP) and Erg6 (anti-Erg6). The positions of molecular size markers are indicated to the left. (E) Yeh2-GFP cofractionates with plasma membrane markers. Membranes from cells expressing Yeh2-GFP (YRS2086) were fractionated on an Accudenz density gradient, and the presence of Yeh2-GFP was detected by Western blotting with an anti-GFP antibody. The distribution of marker proteins in individual fractions from the gradient was determined by Western blotting with antibodies to Erg6, a marker protein for lipid particles (LP) (8); Pho8–anti-Pho8p, a vacuolar phosphatase (VAC); Pma1, the plasma membrane proton pump (PM); Kar2, an ER luminal chaperone (ER) (15); Mnn1, a medial-Golgi α1,3-mannosyltransferase (GOLGI) (20); Sed5, a t-SNARE of the *cis*-Golgi (21); and Tlg1, an endosomal-Golgi t-SNARE (GOLGI/EE) (23). (F) Yeh2-GFP is enriched in the plasma membrane fraction. Plasma membrane was enriched by centrifugation of membranes from cells expressing Yeh2-GFP (YRS2086) on a sucrose step gradient and probed for the presence of the GFP epitope, the GPI-anchored plasma membrane protein Gas1, the lipid particle marker Erg6, the ER luminal protein Kar2, and porin, as a marker protein for mitochondria.

cudenz gradient. The same fractions were also enriched for the plasma membrane proton-pumping ATPase Pma1 (Fig. 5E). Thus, fractionation of Yeh2-GFP expressed from its endogenous promoter is consistent with plasma membrane localization.

To further confirm the localization of Yeh2, plasma membrane from a strain expressing Yeh2-GFP was enriched by fractionation and the relative enrichment of Yeh2-GFP was determined by Western blot analysis. Similar to the glycosylphosphatidylinositol (GPI)-anchored plasma membrane protein Gas1, Yeh2 is enriched about sixfold in the plasma membrane fraction, consistent with plasma membrane localization of Yeh2. Other marker proteins, such as the mitochondrial porin or ER luminal Kar2 or Erg6, however, were not enriched in this fraction (Fig. 5F). These data indicate that all three lipases are membrane associated, that Yeh1 and Tgl1 localize to lipid particles, and that Yeh2 is enriched at the plasma membrane.

A lipase triple mutant has no detectable steryl ester hydrolase activity in vitro. To examine the contribution of the three lipases to steryl ester hydrolysis in vitro, we first determined whether the activity is present in a soluble or membrane-associated form. Therefore, wild-type cells were broken and the homogenate was separated into a soluble fraction and a membrane pellet. Membrane proteins were solubilized with 1% Triton X-100, and the steryl ester hydrolase activity in these fractions was determined by an in vitro assay with cholesterol–[1-^{14}C]oleate as the substrate (46). This analysis revealed that the steryl ester-hydrolyzing activity was detectable in the membrane fraction only, which is consistent with the localization of their tagged versions.

We next determined the steryl ester hydrolase activity in the detergent-solubilized membrane fraction from lipase single-, double-, and triple-mutant cells. This analysis revealed that wild-type cells harbored the highest specific cholesteryl esterase activity whereas the triple mutant was devoid of any detectable activity. The activity present in the single and double mutants indicates that Yeh2 provides the main activity in this in vitro assay, as the activity was reduced to nondetectable levels in strains that lack *YEH2* (Fig. 6A).

Since Yeh1 and Tgl1 are localized to lipid particles, we determined whether cholesteryl esterase activity is present in isolated lipid particles. Consistent with a lipid particle localization for two of these lipases, the specific cholesteryl esterase activity was ~24-fold enriched in the lipid particle fraction from wild-type cells. Analyses of the lipid particle-associated activity from the *yeh1*Δ and *tgl1*Δ single-mutant and *yeh1*Δ *tgl1*Δ double-mutant strains indicate that the detectable activity depends on Tgl1. The in vitro assay hence monitors the activity of Yeh2 and Tgl1 but does not detect any Yeh1-dependent esterase activity (Fig. 6B).

To determine whether the Yeh2-dependent activity is indeed enriched in the plasma membrane, as predicted on the basis of its localization, the cholesteryl esterase activity in plasma membranes from wild-type and *yeh2*Δ mutant cells was determined. Consistent with its localization and its high relative contribution to the total cellular activity, the Yeh2-specific activity was ~10-fold enriched in the plasma membrane fraction and this activity was completely dependent on *YEH2* (Fig. 6C). The results of these in vitro assays are thus consistent with the protein localization data. They also indicate that Yeh1 may require different conditions for its activity in vitro.

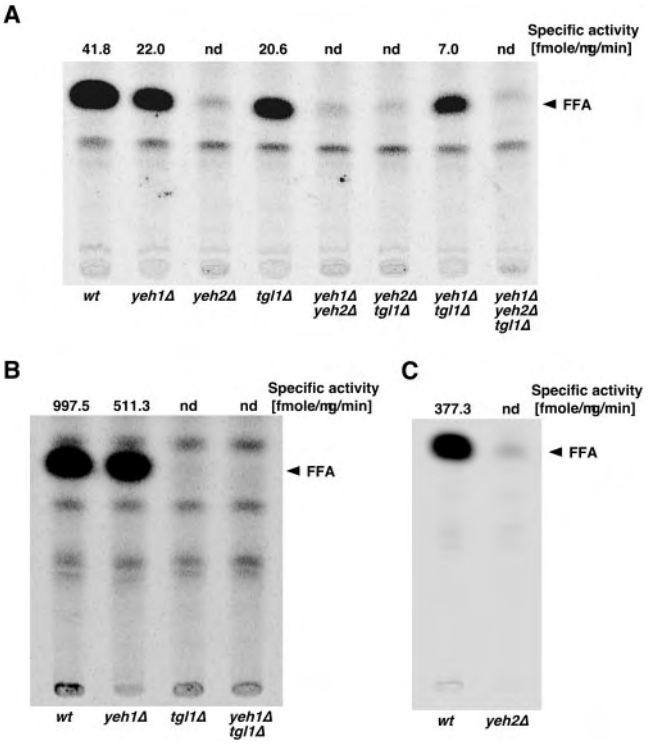


FIG. 6. Lipase triple-mutant cells lack steryl ester hydrolase activity in vitro. (A) In vitro steryl ester hydrolase activity in lipase single-, double-, and triple-mutant cells. Wild-type (*wt*; BY4742, YRS1533) and lipase single (*yeh1*Δ, YRS1972; *yeh2*Δ, YRS1971; *tgl1*Δ, YRS1973)-, double (*yeh1*Δ *tgl1*Δ, YRS1837; *yeh2*Δ *tgl1*Δ, YRS1838; *yeh1*Δ *yeh2*Δ, YRS1948)-, and triple (*yeh1*Δ *yeh2*Δ *tgl1*Δ, YRS1840)-mutant cells were grown in YPD medium and broken with glass beads, and membranes were pelleted. The membrane pellet was solubilized with Triton X-100, and steryl ester hydrolase activity in the detergent extract was determined with cholesterol–[1-^{14}C]oleate as the substrate. The reaction was terminated after 60 min at 30°C, lipids were extracted and separated by TLC, and the released [^{14}C]oleic acid was quantified by radioscanning. The position of the liberated oleic acid is indicated (FFA). The specific activity of steryl ester hydrolase is indicated above the TLC lanes. nd, not detectable. (B) Tgl1-dependent steryl ester hydrolase activity is enriched in lipid particles. Lipid particles from cells of the indicated genotype were isolated, and the steryl ester hydrolase activity was determined in vitro. (C) Yeh2-dependent activity is enriched in the plasma membrane. Plasma membrane from cells of the indicated genotype was isolated, and steryl ester hydrolase activity was determined in vitro.

Overexpression of Yeh1, Yeh2, and Tgl1 confers steryl ester hydrolysis in vivo. To determine whether independent expression of one of the three lipases is sufficient to induce steryl ester mobilization in vivo, expression of these genes was placed under the control of an inducible *GAL1* promoter in a double-mutant background, thus allowing us to monitor the activity of each one of the lipases in the absence of the other two. At the same time, the lipases were tagged at their N termini with GFP, allowing the detection of each of the lipases under repressing (glucose) or inducing (galactose) conditions. Western blot analysis revealed that the GFP-tagged lipases were strongly expressed in cells grown in galactose-containing medium, but the tagged lipases were nondetectable when cells were

grown in glucose-containing medium (Fig. 7A). Examination of the steryl ester pool in the lipase-expressing cells revealed that constitutive expression of each of the three lipases is sufficient to lower the steryl ester pool compared to that in a lipase triple-mutant strain (Fig. 7B). Cells cultivated in glucose, on the other hand, accumulated higher levels of steryl esters than did a corresponding wild-type strain. Levels of triacylglycerols, the second major neutral lipid, however, remained unaffected under these conditions (Fig. 7C). These data thus indicate that the N-terminally GFP-tagged versions of the three lipases are functional and that expression of each one of the three lipases is sufficient to induce mobilization of steryl esters in vivo.

Yeh1, Yeh2, and Tgl1 are integral membrane proteins. To determine whether these lipases fulfill the biochemical properties of integral membrane proteins as predicted on the basis of their sequences and localization (Table 3; Fig. 4 and 5), we examined the solubility of their C-terminally GFP-tagged versions after extraction of membranes with salt (1 M NaCl), alkali (0.1 M Na_2CO_3), or detergent (1% Triton X-100 or 1% SDS). All three lipases remained membrane associated after salt or alkali treatment of membranes but were solubilized by detergents, indicating that they behave as integral membrane proteins (Fig. 8A).

To begin to understand the action of these enzymes on a mechanistic level, their membrane topology with regard to the localization of the active site was characterized in more detail. Therefore, the accessibility of the C-terminal GFP to cleavage by proteinase K was examined. Incubation of the 13,000 × *g* membrane pellet from strains expressing Yeh1-GFP or Tgl1-GFP with increasing concentrations of proteinase K revealed that the C termini of both proteins are readily degraded (Fig. 8B). Probing of these Western blots with an antibody against the luminal protein Kar2 indicates that the membrane seal remained intact during these incubations. These results thus indicate that the C termini of Yeh1 and Tgl1 are cytosolic.

To determine the accessibility of the C terminus of Yeh2, enriched plasma membrane vesicles from Yeh2-GFP-expressing cells were incubated with proteinase K. This resulted in the rapid appearance of a faster-migrating cleavage product that appeared to be the result of the removal of an ~17-kDa fragment from the N-terminal domain of Yeh2-GFP. Blotting of these membranes with an antibody against the GPI-anchored protein Gas1 revealed that Gas1 was protease protected, indicating that the enriched plasma membrane mainly consisted of inside-out vesicles and hence that the protected C terminus of Yeh2 is likely localized in a luminal-extracellular compartment.

To further refine this topological characterization, the accessibility of the N termini of the three lipases to protease was examined. Therefore, the lipases were tagged with GFP at their N termini and their expression was placed under the control of the *GAL1* promoter. Microscopic examination of cells expressing the N-terminally GFP-tagged lipases after overnight growth in galactose-containing medium revealed lipid particle staining for both GFP-Yeh1 and GFP-Tgl1 and plasma membrane staining for GFP-Yeh2 (data not shown). Proteinase accessibility of the N-terminal GFP was determined with the 13,000 × *g* membrane pellet from GFP-Yeh1- and GFP-Tgl1-expressing cells. These analyses revealed that the N-terminal epitope of Yeh1 was rapidly removed by the protease (Fig. 8C). Protease

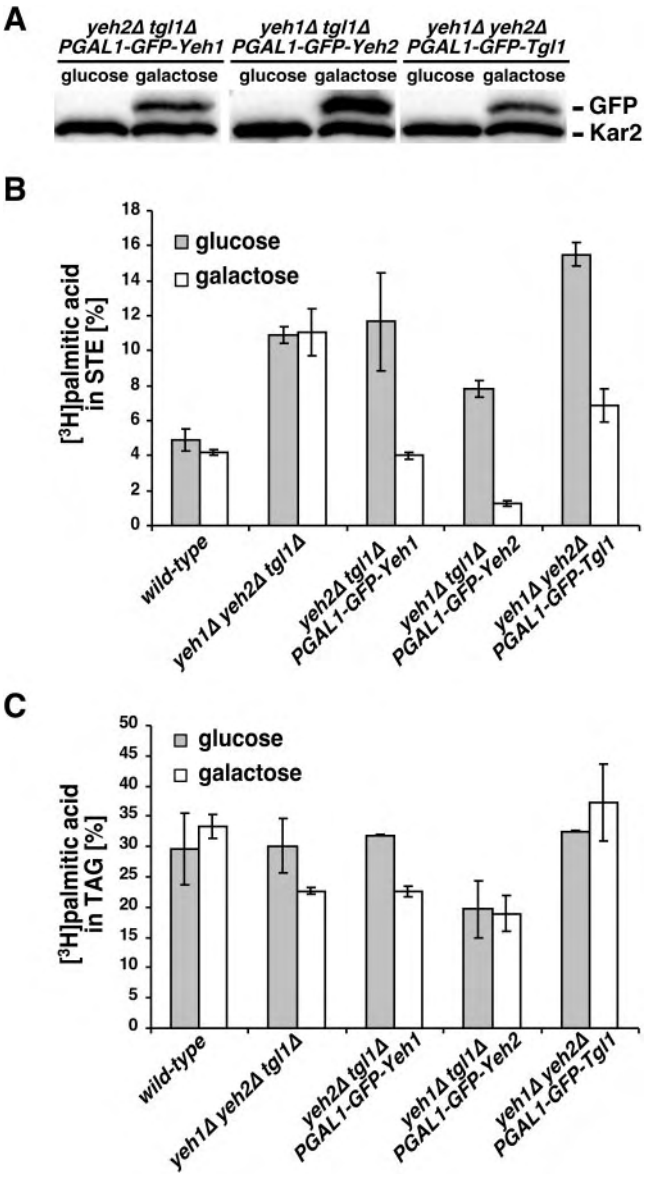


FIG. 7. Overexpression of N-terminally tagged lipases is sufficient to reduce steryl ester levels. (A) Overexpression of the lipases in a lipase double-mutant background. Strains (YRS2161, YRS2162, and YRS2163) that express an N-terminally GFP-tagged lipase under the control of a galactose-inducible promoter in the absence of the other two lipases were cultivated in either glucose- or galactose-containing rich medium for 16 h. Proteins were extracted, and equal amounts were subjected to Western blot analysis with an antibody against GFP and ER luminal Kar2. (B) Overexpression of GFP-Yeh1, GFP-Yeh2, and GFP-Tgl1 reduces steryl ester but not triacylglycerol levels in vivo. Cells of the indicated genotype were cultivated in either glucose- or galactose-containing rich medium and labeled with [^{3}H]palmitic acid for 16 h. The relative proportions of [^{3}H]palmitic acid in the steryl ester (STE) (B) and triacylglycerol (TAG) (C) pools were quantified by radioscanning of TLC plates. Values represent means and standard deviations of two independent experiments.

cleavage of GFP-Tgl1, on the other hand, resulted in the rapid appearance of a faster-migrating product, indicating that the N terminus of GFP-Tgl1 is protected but that its C terminus is cleaved to release a fragment of ~12 kDa. Accessibility of the N terminus of Yeh2 was assessed again with enriched plasma

TABLE 3. Characteristics of the three predicted lipases

Lipase	Molecular mass (kDa)	Signal sequence[a]	Signal sequence[b]	TMD[c]	TMD[d]	TMD[e]	Potential N-glycosylation site (position)[f]
Yeh1	66.51	31	32	8–36 392–415	13–32 342–363 390–412	12–36	139 140 419
Yeh2	62.45		Signal anchor	11–35 361–389	12–31 364–385	11–31 361–379 399–416	105
Tgl1	62.98	28	None	4–32 243–271 354–381	11–30 195–213 249–268	14–33	46 424 536

[a] The signal sequences shown are indicated by the yeast genome database (http://db.yeastgenome.org) and were determined by using the application sigcleave available in the EMBOSS package (http://emboss.sourceforge.net/).
[b] Signal sequence derived by SignalP 3.0 (http://www.cbs.dtu.dk/services/SignalP/).
[c] Transmembrane domains (TMDs) indicated by the yeast genome database on the basis of tmap applications available in the EMBOSS package.
[d] TMDs derived by TMpred (http://www.ch.embnet.org/software/TMPRED_form.html).
[e] TMDs derived by Phobius (http://phobius.cgb.ki.se/).
[f] The numbers and amino acid positions of potential N-linked glycosylation sites were predicted by using the NetNGlyc 1.0 server (http://www.cbs.dtu.dk/services/NetNGlyc/).

membrane vesicles. Incubation of the plasma membrane from GFP-Yeh2-expressing cells with protease resulted in rapid disappearance of GFP-Yeh2, indicating that the N terminus of Yeh2 is cytosolic.

To determine whether the potential N-glycosylation sites of the three lipases are accessible for ER luminal glycosylation, the C-terminally GFP-tagged versions of these proteins were subjected to treatment with endoglycosidase H and their electrophoretic mobility was compared with that of nontreated controls. The results of this analysis did not reveal any difference between treated and untreated samples, indicating that these proteins are not N-glycosylated and hence that the corresponding asparagine residues are not exposed to the ER lumen. Probing the same Western membranes with an antibody against a protein known to be N-glycosylated, Gpi8, indicated that the endoglycosidase H treatment was successful (data not shown).

Taken together, these results indicate that Yeh1 is a membrane protein that localizes to intracellular lipid particles with both N and C termini exposed to the cytosol, which suggests that the protein is anchored in the membrane by at least two transmembrane domains. Because lipases contain an alpha/beta hydrolase fold, whereas known integral membrane proteins contain either alpha-helical or beta-barrel structures in their membrane-embedded domain, we propose that Yeh1 has a hairpin-like transmembrane domain in its N-terminal region that anchors the protein to the membrane. This would allow both termini of Yeh1 to face the same compartment and would allow proper folding of the lipase domain. A hairpin-like membrane anchor is supported by the fact that one of the algorithms used to predict transmembrane regions, Phobius, indicates that the N terminus of Yeh1 has a particularly long hydrophobic stretch encompassing amino acids 12 to 36 (Table 3). A similar hairpin-like topology has been suggested for caveolin (18); in this case, Phobius predicts a membrane anchor of 23 amino acids, which is close to the 24 residues predicted for Yeh1.

Tgl1, which also localizes to lipid particles, has its C terminus exposed to the cytosol and the N terminus protected in a lumen compartment. Such a topology could be due to either one or three transmembrane domains. As the topology with three transmembrane domains would place the hydrolase domain into a transmembrane segment, we propose a type I topology for Tgl1 as the more plausible alternative. Yeh2 also has its N and C termini at different sites of the membrane, compatible with either one or three transmembrane domains. On the basis of the same arguments, we propose the simpler type II topology for Yeh2. A schematic overview of the proposed membrane topology of the three proteins is shown in Fig. 9. All three topologies are compatible with a potential alpha/beta hydrolase fold of the respective lipase.

DISCUSSION

As an important step toward understanding sterol homeostasis at the cellular level, the aim of this study was to identify the steryl ester-hydrolyzing enzyme(s) of yeast. By a candidate gene approach, we identified a family of potential lipases with a predicted alpha/beta hydrolase-associated lipase domain as being required for steryl ester hydrolysis in vivo and in vitro.

The three lipases encoded by *YEH1*, *YEH2*, and *TGL1* constitute the yeast paralogues of the mammalian acid lipase family, which includes lysosomal acid lipase A (LIPA), the enteric gastric/lingual lipase LIPF, and four novel members that are predicted on the basis of the human genome sequence, LIPL1 to LIPL4 (3, 5). The lysosomal acid lipase is a key enzyme in the intracellular degradation of neutral lipids that have been internalized through receptor-mediated endocytosis of lipoprotein particles (19). The enzyme hydrolyzes cholesteryl esters and triacylglycerols, releasing free cholesterol and fatty acids. The released cholesterol rapidly equilibrates with the cellular pool to regulate its endogenous synthesis, esterification, and receptor-mediated uptake (11). Feedback regulation of sterol synthesis by the steryl ester pool in yeast is poorly characterized, but the lipase mutants now provide an important tool to address this pathway genetically. LIPA is active toward both steryl esters and triacylglycerols (5); the yeast enzymes, on the other hand, appear to be more specific for steryl esters, as triacylglycerol mobilization in vivo is not affected in triple-mutant cells.

LIPA deficiency in humans causes two rare autosomal re-

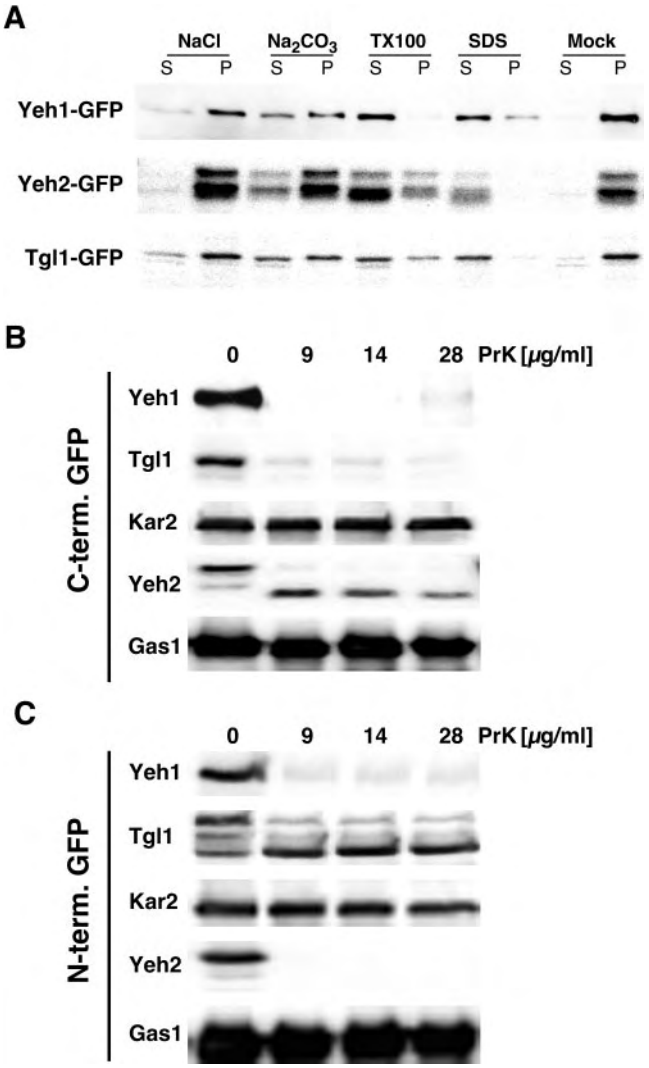


FIG. 8. Yeh1, Yeh2, and Tgl1 are integral membrane proteins. (A) Yeh1-, Yeh2-, and Tgl1-GFP are solubilized by detergents. Strains expressing the C-terminally (C-term.) tagged lipases (Yeh1-GFP, YRS1974; Yeh2-GFP, YRS2986; Tgl1-GFP, YRS1858) were grown in YPD medium, cells were broken with glass beads, and membranes were pelleted by centrifugation at 13,000 × *g*. Fifty micrograms of protein from the membrane pellet was incubated for 30 min at 4°C with 1 M NaCl, 0.1 M Na_2CO_3, 1% Triton X-100 (TX100), 1% SDS, or buffer alone (Mock) and then centrifuged at 13,000 × *g* for 30 min to yield soluble (S) and pellet (P) fractions. Proteins were precipitated by TCA, and 10 μg was separated by electrophoresis and probed for the presence of the GFP epitope with an anti-GFP antibody. (B) Protease sensitivity of the C-terminal GFP on Yeh1, Yeh2, and Tgl1. Fifty micrograms of protein from either the 13,000 × *g* membrane pellet (Yeh1-GFP and Tgl1-GFP) or the enriched plasma membrane fraction (Yeh2-GFP) was incubated with the indicated proteinase K (PrK) concentration for 30 min on ice. Proteins were precipitated by TCA, and 10 μg was separated by electrophoresis and probed for the presence of the GFP epitope or for Kar2 and Gas1, respectively. (C) Protease sensitivity of the N-terminal (N-term.) GFP on Yeh1, Yeh2, and Tgl1. Strains expressing the N-terminally tagged lipases (GFP-Yeh1, YRS2083; GFP-Yeh2, YRS2085; GFP-Tgl1, YRS2084) were grown in YPGal medium, cells were broken with glass beads, and proteins from either the 13,000 × *g* membrane pellet (GFP-Yeh1 and GFP-Tgl1) or the enriched plasma membrane fraction (GFP-Yeh2) were incubated with the indicated proteinase K concentration for 30 min on ice. Proteins were precipitated by TCA, separated by electrophoresis, and probed for the presence of the GFP epitope and for Kar2 and Gas1, respectively.

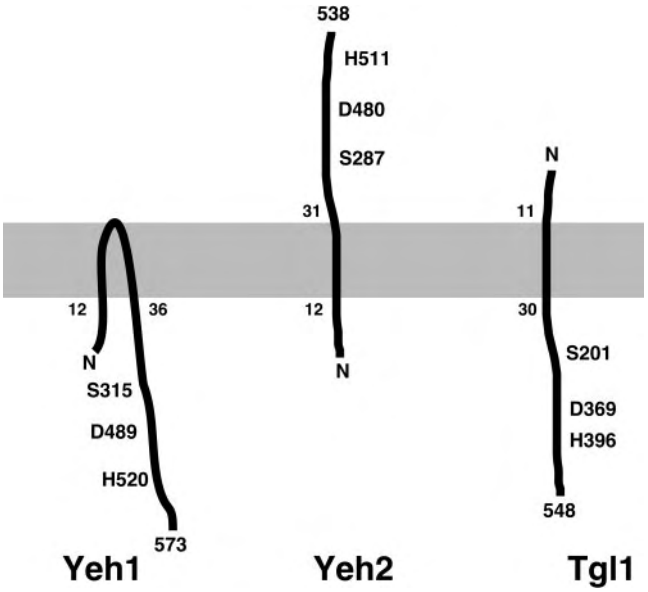


FIG. 9. Schematic representation of the proposed membrane topology of yeast lipases Yeh1, Yeh2, and Tgl1 with regard to the catalytic-triad residues.

cessive disorders, Wolman disease and cholesterol ester storage disease (4, 6, 13, 41). Wolman disease is lethal within the first year of life because of hepatosplenomegaly, adrenal calcification, and massive accumulation of triglycerides and cholesteryl esters in these organs, as well as in macrophages and blood vessels. Cholesterol ester storage disease is a less severe disorder with longer survival, hepatomegaly, premature atherosclerosis, and dyslipoproteinemias. Patients with the milder form of this lipid storage disease retain some residual LIPA activity (39). The crucial role of LIPA in steryl ester metabolism also prompted its consideration as an important factor in atherogenesis (6).

LIPA localizes to endosomes, where it cleaves low-density-lipoprotein-derived substrates. Our subcellular localization of GFP-tagged versions of the three yeast lipases indicates that Yeh1 and Tgl1 both localize to lipid particles whereas Yeh2 is enriched in the plasma membrane. Lipid particle localization of two of the three yeast steryl ester hydrolases, Yeh1 and Tgl1, is likely to allow regulated access to their neutral lipid substrates, which are stored in these particles (29). Intriguingly, however, both lipases appear to be membrane proteins on the basis of the detergent requirement for their solubilization. Yeh1 and Tgl1 are each proposed to be an integral membrane protein with an N-terminal transmembrane segment(s). This membrane anchoring is particularly remarkable in light of the fact that the lipid particle membrane has been proposed to be a monolayer rather than a normal bilayer membrane (29). It would thus be interesting to examine how these transmembrane domains can be accommodated in a lipid monolayer. Alternatively, it might be conceivable that the two lipases localize to domains of the lipid particle membrane that retain a bilayer rather than a monolayer structure.

The role of Yeh2 in light of its plasma membrane localization is less obvious than that of lipid particle-localized Yeh1 and Tgl1. But given the fact that the active site of the enzyme

is located in a luminal-extracellular compartment, it is tempting to speculate that Yeh2 may be important to cleave either extracellular or endocytosed steryl esters. While yeast readily takes up exogenous fatty acids, sterols are taken up only under anaerobic conditions (32). The observation that Yeh2 alone in a *yeh1*Δ *tgl1*Δ double mutant does not efficiently mobilize steryl esters in vivo is consistent with a limiting access of the enzyme to the intracellular steryl ester pool, as predicted on the basis of the localization and topology of the enzyme. It is interesting that Yeh2 binds phosphatidylinositol-4,5-bisphosphate and phosphatidylinositol-3,4,5-triphosphate in vitro (54). While phosphatidylinositol-3,4,5-triphosphate has not been detected in *S. cerevisiae* (35, 36), it remains to be seen whether these signaling lipids affect the activity of the enzyme. The size variants of Yeh2 detected on Western blots are likely to be due to O-linked glycosylation of the luminal domain of Yeh2, which would be consistent with the proposed type II topology of the enzyme.

Levels of steryl esters are coordinated with the growth phase (9, 48). It is thus necessary that their synthesis, mobilization, or both processes are tightly regulated. An interesting question for further studies will thus be to examine whether and, if so, how the lipases obtain regulated access to their substrates and to further investigate the molecular mechanism of this regulation. Mobilization of steryl esters from lipid particles requires energy and ongoing protein synthesis but is independent of microtubules (28). Recent results obtained with mammalian cells suggest that an interplay between components that localize to the lipid droplets, such as perilipin and the lipase, in this case HSL, is important to coordinate substrate access. This interplay is regulated by protein kinase A to increase the rate of lipolysis by 30- to 100-fold (44). Even though yeast lipid particles lack any obvious perilipin orthologue, it is interesting that both Yeh1 and Tgl1, but not Yeh2, contain potential cyclic-AMP-dependent protein kinase A phosphorylation sites. Taken together, the identification and topological characterization of three yeast lipases now provide the opportunity to use *S. cerevisiae* to further characterize the molecular mechanisms that control sterol homeostasis at the cellular level.

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Additional Experiments

Additional experiments Part I: Drugs and phenotypes

1. Introduction

STE synthesis and hydrolysis is coordinated with the growth phase and STE start to accumulate at the beginning of the stationary phase (1). The importance of STE mobilization for sterol homeostasis remains unknown, but the identification of three yeast STE hydrolases encoded by *YEH1*, *YEH2*, and *TGL1* enabled us to examine cellular sterol homeostasis in more detail. Interestingly, synthesis of STE in not essential and also abolishing STE hydrolysis in a triple-mutant *yeh1Δ yeh2Δ tlg1Δ* leads to no obvious phenotype under standard growth conditions (2, 3). However, it has been shown earlier that the ability to accumulate storage lipids influences long-term survival of stationary phase cells, as mutants deficient for neutral lipid synthesis exhibit reduced survival during nitrogen starvation (3). Neutral lipids, such as STE and TAG, serve as a storage form for energy and particularly STE are an important source for sterols upon acute demand. Thus, STE mobilization is expected to be important for cellular sterol homeostasis. To study the physiological role of the STE pool we used a set of single, double, and triple STE hydrolase deletion strains. These deletion mutants were phenotypically characterized for sensitivity to drugs that block endogenous ergosterol biosynthesis, and for long-term survival during stationary phase to determine the consequences of a partial or complete loss of STE mobilization.

2. Materials and Methods

2.1. Strains, media, and growth conditions

Yeast strains used in this study are listed in Table 1. Single deletion mutants were obtained from EUROSCARF (www.rz.unifrankfurt.de/FB/fb16/mikro/euroscarf/index.html), and double and triple-mutant strains were generated by gene disruption with PCR deletion cassettes or by crossing of single-mutants (5). For plate assays on drug containing media, fresh overnight cultures were inoculated into YPD (1% Bacto Yeast Extract, 2% Bacto Peptone [U.S. Biological, Swampscott, Mass.], 2% glucose) to OD_{600} of 0.2 and grown to OD_{600} of 1 at 24°C or 30°C. Next, 2 OD_{600} units were harvested, resuspended in 200 µl fresh YPD and 10-fold serial dilutions were spotted on agar plates with or without inhibitors. The agar plates were prepared by adding the drugs from stock solutions to molten agar equilibrated to 50 °C. Agar plates containing the inhibitors terbinafine (5 to 100 µg/ml) and cerulenine (0.5 to 10 µg/ml) were buffered with 50 mM Citrate-Pi to pH 6. For the drugs fenpropimorph (2.3 µM and 4.69 µM) and nystatin (0.5 to 5 µg/ml) unbuffered agar plates

were used. Cell growth was examined after 3 to 4 days incubation at 24°C and 30 °C. All experiments were performed in duplicates.

Table 1: *Saccharomyces cerevisiae* strains used in this study

Strain	Relevant genotype	Source or reference
YRS1533	BY4742; *MATα his3Δ1 leu2Δ0 ura30 lys2Δ0*	EUROSCARF; 18
YRS1972	*MATα his3Δ1 leu2Δ0 ura30 lys2Δ0 yeh1*::*kanMX4*	EUROSCARF; 18
YRS1971	*MATα his3Δ1 leu2Δ0 ura30 lys2Δ0 yeh2*::*kanMX4*	EUROSCARF; 18
YRS1973	*MATα his3Δ1 leu2Δ0 ura30 lys2Δ0 tg11*::*kanMX4*	EUROSCARF; 18
YRS1948	*MATα his3Δ1 leu2Δ0 ura30 lys2Δ0 yeh1*::*HIS3MX6 yeh2*::*kanMX4*	This study; 2
YRS1837	*MATα his3Δ1 leu2Δ0 ura30 lys2Δ0 yeh1*::*HIS3MX6 tg11*::*kanMX4*	This study; 2
YRS1838	*MAT? his3Δ1 leu2Δ0 ura3Δ0 met15Δ0 LYS2 yeh2::kanMX4 tg11::kanMX4*	This study; 2
YRS1840	*MAT? his3Δ1 leu2Δ0 ura3Δ0 met15Δ0 LYS2 yeh1::HIS3MX6 yeh2::kanMX4 tg11::kanMX4*	This study; 2
H1246	*MATα are1::HIS3 are2::LEU2 dga1::KanMX4 lro1::TRP1 ADE2*	3

2.2. Growth recovery assay – viability during stationary phase

Initially, cells were grown overnight in YPD, diluted 1:100 in 50 ml fresh YPD, and grown to stationary phase for 5-7days at 24°C. After reaching stationary phase, equivalents of 50 OD_{600} were pelleted (3,000 rpm for 5 min at 24°C) and washed twice with 1 M sorbitol and once with H_2O. Next, cells were resuspended in 10 ml H_2O and further incubated at 24°C with gentle shaking for two days. This step is necessary to ensure that the cells are not growing in water anymore. After this step, aliquots (equivalents of 1 OD_{600}) were removed every second day over a period of 30 days. These aliquots were plated on YPD agar and incubated at 30°C to define the number of colony forming units (CFU) that remained as a function of time in water. In addition, aliquots at each time point were checked for cell viability which was determined by incubation with 0.1% methylene blue (Sigma) and counting of blue (dead) cells. All experiments were performed in duplicates for each time point.

3. Results

3.1. Strains with impaired STE hydrolysis are sensitive to inhibitors of the ergosterol biosynthetic pathway.

Terbinafine and fenpropimorph are two important antimycotic drugs which block ergosterol biosynthesis. Terbinafine belongs to a class of antifungal compounds called allylamines and inhibits the fungal squalene epoxidase (Erg1p) (6). Erg1p catalyzes the epoxidation of squalene to 2,3-oxidosqualene, one of the final steps in ergosterol biosynthesis (7). Yeast cells treated with terbinafine accumulate squalene and are depleted for ergosterol. Fenpropimorph is another important antifungal drug which belongs to the group of morpholines. The target enzymes of fenpropimorph are the C-8 sterol isomerase (Erg2p) and C-14 sterol reductase (Erg24p), which act downstream of Erg1p in the ergosterol biosynthetic pathway (8, 9). The *ERG24* gene encodes the enzyme which converts 4,4-dimethylcholesta-8,14,24-trienol to 4,4 dimethylcholesta-8,24-dienol, whereas Erg2p converts fecosterol to episterol in later steps of the pathway (10). Treatment of yeast with terbinafine or fenpropimorph leads to depletion of ergosterol and to growth inhibition. If one keeps in mind that yeast stores STE in lipid particles as a source for sterols upon demand, it can be speculated that cells which are able to mobilize STE from lipid particles should be less sensitive to treatment with these antifungal compounds as compared to strains which are deficient for STE hydrolysis. To test this hypothesis we examined the growth of single, double, and triple STE hydrolase deletion strains on media containing terbinafine and fenpropimorph. A lipase triple-mutant that is completely deficient for STE hydrolysis is slightly more sensitive to terbinafine at drug concentrations of 30 µg/ml and 50 µg/ml than a wild-type strain (Fig.1A). Interestingly, growth of STE hydrolase double-mutants seems not to be affected by terbinafine treatment (Fig.1A). Single-deletion lipase mutants were not tested on YPD with terbinafine, because the inhibitory effect on growth of double-deletion mutants with one remaining STE hydrolase was not satisfying, and thus it is unlikely that terbinafine could inhibit growth of a single-mutant where two of three STE hydrolase are still present. Treatment of lipase mutants with fenpropimorph shows that the *yeh1Δ yeh2Δ tgl1Δ* triple-mutant is highly sensitive to this antifungal compound (Fig.1B). Growth inhibition with fenpropimorph is even more pronounced than that observed with terbinafine. Moreover, already a *yeh2Δ* single-mutant is sensitive to treatment with 4.69 µM fenpropimorph. Single deletion of *TGL1* or *YEH1*, on the other hand, does not lead to such strong inhibition even though growth is slightly inhibited in these mutants compared to wild-type (Fig.1B). An interesting observation is that the growth of lipase double-mutants on fenpropimorph media is again comparable to wild-type.

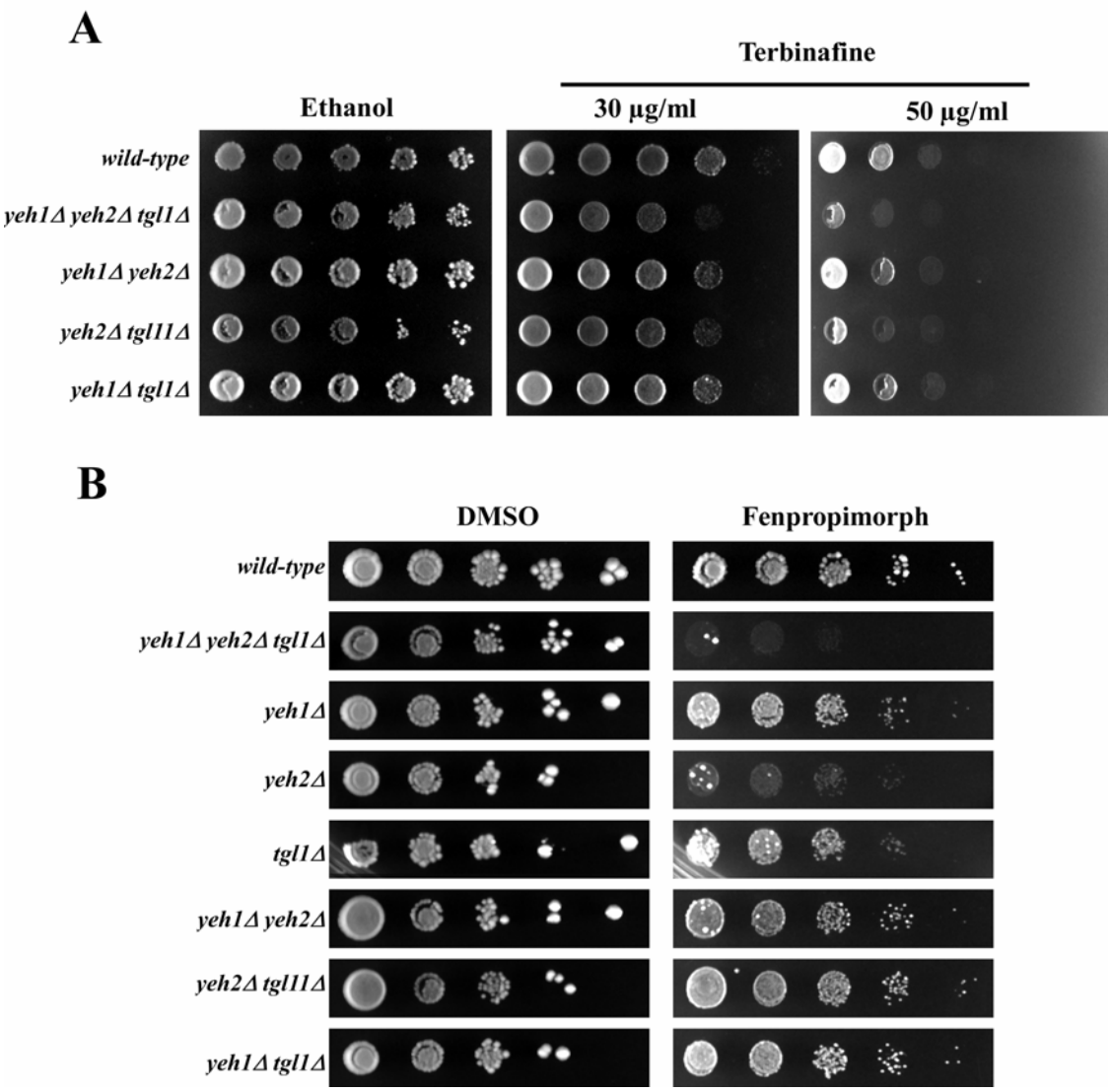

Figure 1: Steryl ester hydrolase mutants are sensitive to inhibitors of ergosterol biosynthesis. (A) Wild-type (BY4742, YRS1533), *yeh1Δ yeh2Δ tgl1Δ* (YRS1840), *yeh1Δ yeh2Δ* (YRS1948), *yeh2Δ tgl1Δ* (YRS1838) and *yeh1Δ tgl1Δ* (YRS1837) mutant cells were spotted onto YPD media containing no inhibitor, or 30 µg/ml or 50 µg/ml terbinafine. Images of plates were taken after 3 days of incubation at 30°C. (B) Lipase single-mutant (*yeh1Δ*, YRS1972; *yeh2Δ*, YRS1971; *tgl1Δ*, YRS1973), double-mutant (*yeh1Δ yeh2Δ*, YRS1948; *yeh2Δ tgl1Δ*, YRS1838; *yeh1Δ tgl1Δ*, YRS1837) and triple-mutant (*yeh1Δ yeh2Δ tgl1Δ*, YRS1840) cells were spotted and grown on media containing 4.69 µM fenpropimorph. Growth inhibiton of fenpropimorph was examined after 3 to 4 days incubation at 30°C.

This effect was observed on terbinafine containing media before (Fig1.A) and suggests that if two of three lipases are missing then the remaining STE hydrolase may compensate for their function. On the other hand, growth of STE hydrolase mutants is not affected by 0.5 µg/ml cerulenin, whereas an *are1Δ are2Δ dga1Δ lro1Δ* quadruple -mutant that lacks lipid particles is highly sensitive to the drug (Fig.2A).

Higher concentration of cerulenin (2 µg/ml to 10 µg/ml) completely inhibits growth of all strains. Cerulenin is an antibiotic which irreversibly inhibits the fatty acid synthetase thereby depleting cells for fatty acids (11). The strong effect of cerulenin on the growth of an *are1Δ are2Δ dga1Δ lro1Δ* mutant shows the importance of lipid particles as a source of not only sterols but also fatty acids. The resistance of a strain deficient for STE mobilization to cerulenin suggests that STE, which are composed of a fatty acid esterified to the 3-OH group of sterol, are more likely the source for free sterols rather then for fatty acids. Taken together, our results show that a strain deficient for STE hydrolysis is sensitive to antifungal compounds which inhibit ergosterol biosynthesis, indicating that STE mobilization is important for growth under sterol depleting conditions.

3.2. Steryl ester hydrolase mutants are resistant to nystatin and thus seem to have altered sterol composition and/or perturbations of the plasma membrane.

Nystatin belongs to the polyene antibiotics and is widely used for treatment of fungal infections and mycoses. The mechanism of action of nystatin is thought to be due to an impairment of membrane function by interaction with ergosterol. Thus, the antifungal activity of this drug is dependent on the membrane composition. Nystatin resistant mutants were found to have altered plasma membrane composition and reduced ergosterol content (12). Not surprisingly, mutants in the ergosterol biosynthetic pathway show resistance to nystatin toxicity, which is thought to be the consequence of the absence or low levels of ergosterol in the plasma membrane of these mutants (13). Testing the growth of STE hydrolase mutant strains on nystatin containing media revealed that these mutants are also resistant to this antifungal drug (Fig.2B). Growth of wild-type is inhibited already at concentrations of 0.5 µg/ml nystatin. Interestingly, single deletion of any of the three STE hydrolases is sufficient to render these mutants resistant to nystatin. All double-mutants and the *yeh1Δ yeh2Δ tgl1Δ* triple-mutant show the same growth resistance as observed for single deletion strains. Only the *yeh1Δ yeh2Δ* double-mutant seems to be less resistant to nystatin treatment. In conclusion, the experiment shows that lack of any STE hydrolase leads to growth resistance on nystatin. Furthermore, the results lead to the hypothesis that mutants with partial or fully blocked STE mobilization have altered (lower) levels of ergosterol in the plasma membrane.

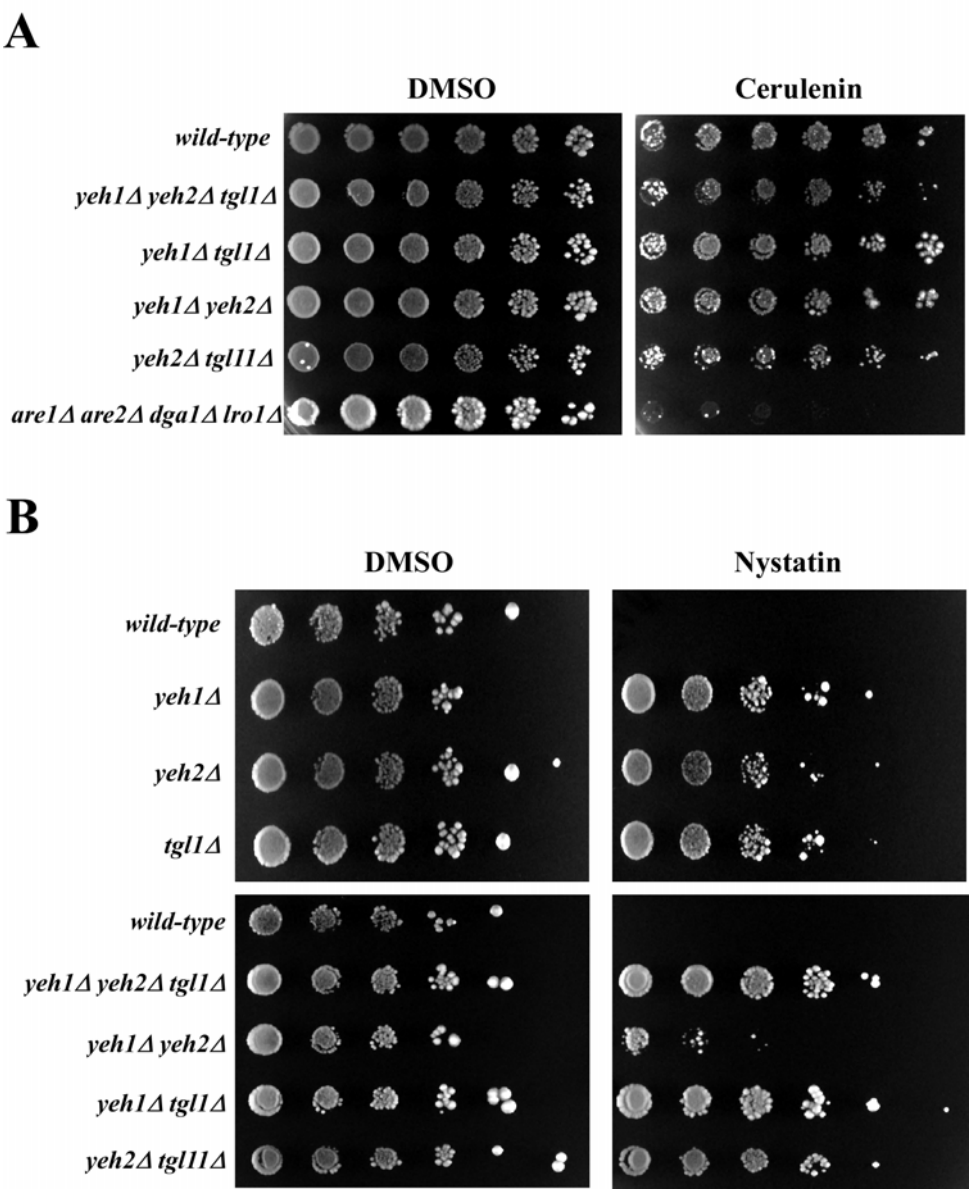

Figure 2: Steryl ester hydrolase mutants are not sensitive to cerulenine and resistant to nystatin. (A) Lipase double-mutant (*yeh1Δ yeh2Δ*, YRS1948; *yeh2Δ tgl1Δ*, YRS1838; *yeh1Δ tgl1Δ*, YRS1837) and triple-mutant (*yeh1Δ yeh2Δ tgl1Δ*, YRS1840) cells were grown on YPD media containing 0.5 µg/ml cerulenin. As a control growth inhibition of a neutral lipid deficient strain (*are1Δ are2Δ dga1Δ lro1Δ*, H1246) is shown. (B) Growth of wild-type (BY4742, YRS1533), lipase single-mutant (*yeh1Δ*, YRS1972; *yeh2Δ*, YRS1971; *tgl1Δ*, YRS1973), lipase double-mutant (*yeh1Δ yeh2Δ*, YRS1948; *yeh2Δ tgl1Δ*, YRS1838; *yeh1Δ tgl1Δ*, YRS1837) and lipase triple-mutant (*yeh1Δ yeh2Δ tgl1Δ*, YRS1840) cells was examined on media containing 0.5 µg/ml nystatin after 4 days of incubation at 30°C.

3.3. A *yeh1Δ yeh2Δ tgl1Δ* triple-mutant looses viability during stationary phase

Neutral lipids are an important source for sterols and fatty acids upon rapid demand. STE serve as a storage form for sterols and fatty acids and start to accumulate when yeast enters the stationary growth phase (1). Upon demand, e.g. if the cells “restart” growth from

stationary phase, STE are hydrolyzed to supply the need for free sterols of growing membranes and possible other cellular needs. This hypothesis awaits final confirmation. Neutral lipid synthesis is known to be dispensable under standard growth conditions, but an *are1Δ are2Δ dga1Δ lro1Δ* quadruple-mutant which is deficient for neutral lipid synthesis shows reduced long-term survival in stationary phase during nitrogen starvation (2). Mobilization of TAG is catalyzed by three TAG lipases, namely Tgl3p, Tgl4p, and Tgl5p, whereas hydrolysis of STE is catalysed by three STE hydrolases encoded by *YEH1*, *YEH2*, and *TGL1*, which haven been described recently (15, 16, 3). To examine the need of STE mobilization for growth in yeast, we took advantage of a *yeh1Δ yeh2Δ tgl1Δ* triple-mutant, which is deficient for STE hydrolysis. The idea was to grow these mutant cells in rich media to growth arrested-phase (stationary phase) and to allow them to accumulate STE. The stationary phase cells were then shifted from rich media to water in order to deplete them for nutrients. Wild-type and STE hydrolase triple-mutant cells were kept in water for up to 25 days and the colony forming potential of the strains was examined as a function of time spent in water. Practically, this was done by transferring aliquots of cells at indicated time points (every second or third day) to fresh rich media, thereby allowing the cells to restart growth. The result of this experiment was that the STE hydrolase deleted mutant showed a dramatic loss of colony forming units (CFU) which started after the 5^{th} day (Fig.3). The CFU rapidly decreased further and finally, between the 14^{th} and 17^{th} day, the triple-mutant completely failed to grow when transferred to fresh media. In contrast, a wild-type strain exhibited constant colony forming potential during a time period of 25 days in water. Single and double STE hydrolase deletion strains were also tested, but none of them showed any decrease of CFU during 25 days in water (data not shown). To test if triple-mutant cells are still viable in water but are unable to restart growth, or if they are dead, methylene blue staining was used. This assay is based on the observation that viable cells exclude the dye from the cytoplasm and remain colourless while dead or permeable cells stain blue (14). Analysis of viability with methylene blue of the *yeh1Δ yeh2Δ tgl1Δ* triple-mutant showed that after 14 days in water approximately 30% of the cells were dead and this number increased up to 59% dead cells in the following 5 days. In contrast to this, a wild-type strain showed 5% dead cells after 14 days in water and the number of dead cells did not increase as drastically as it was observed for the triple STE hydrolase mutant (data not shown). It has to be mentioned here, that the experiment was repeated once again in duplicates. This time the triple-mutant showed reduced colony forming potential over time but not as drastically as in the first experiment.

Additional repetition of the experiments is thus necessary to finally confirm that a triple STE hydrolases mutant looses viability during stationary growth phase.

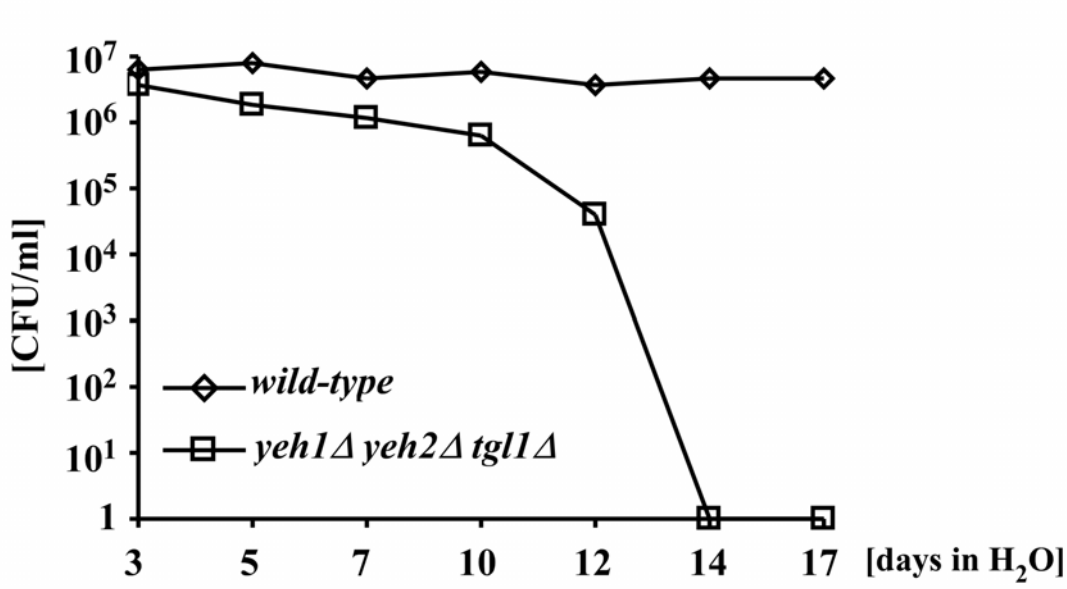

Figure 3: A strain deficient for STE hydrolysis loses viability during stationary phase. Wild-type (BY4742, YRS1533) and *yeh1Δ yeh2Δ tgl1Δ* (YRS1840) mutant cells were first grown to stationary phase and 50 OD_{600} units were transferred to H_2O. During incubation for 17 days at 24°C, aliquots were removed every second or third day and cells were plated onto YPD plates to determine the CFU/ml.

4. Discussion

In this work we examined the physiological role of neutral lipids, especially that of the STE pool. Previous work using a strain deficient for neutral lipid synthesis already showed that, although neutral lipid synthesis is not essential under standard conditions, this strain exhibits loss of viability during stationary phase (3). Neutral lipids are composed of TAG and STE, which are stored in lipid particles in yeast (17). A strain deficient for neutral lipid synthesis thus affects both, the TAG and the STE pool. In our work, we wanted to elucidate the role of the STE pool only and its implication on growth since STE mobilization is thought to be important to support rapid initiation of cellular growth and division (1). In yeast there exists a family of three STE hydrolases named Yeh1p, Yeh2p, and Tgl1p (3). To investigate the importance of STE hydrolysis we took advantage of a set of single, double, and triple STE hydrolase mutants, which are partially or completely deficient for STE mobilization. First we examined the effects of blocking ergosterol biosynthesis in these mutant strains. The experiment showed that terbinafine and fenpropimorph, two well characterized inhibitors of ergosterol biosynthesis, inhibit the growth of a *yeh1Δ yeh2Δ tgl1Δ* triple-mutant, although to

different extend. The lipase triple-mutant is slightly sensitive to terbinafine and highly sensitive to fenpropimorph. It is not known why these two antifungal compounds differ in their action. Interestingly, already single deletion of *YEH2* leads to sensitivity to fenpropimorph. This is in contrast to deletion of the other two STE hydrolases, *YEH1* and *TGL1*, as well as to the double STE hydrolase deletion mutants, which show growth comparable to wild-type on fenpropimorph. However, the results indicate once more the important role of STE as a source for sterols within the cells and even more importantly confirms that STE hydrolysis is needed to support growth when the endogenous ergosterol pool gets depleted. In line with the role of STE as a storage form for sterols, we also found that none of the STE hydrolase mutants shows increased sensitivity against cerulenin. Cerulenin, an inhibitor of fatty acid synthesis, depletes yeast for fatty acids and inhibits growth. An *are1Δ are2Δ dga1Δ lro1Δ* quadruple-mutant, which is deleted for all STE and TAG synthesizing genes, is very susceptible to this drug. Thus, one could speculate that upon fatty acid depletion the esterified fatty acid in the STE would partially contribute to growth. This seems not to be the case because otherwise at least a mutant deleted for all three STE hydrolases, should be susceptible to cerulenin. STE may therefore constitute a storage form primarily for sterols and not for fatty acids. The main source of fatty acids upon demand is likely TAG, the second neutral lipid in yeast, because hydrolysis of one molecule of TAG results in liberation of three fatty acids as compared to only one fatty acid which gets released upon STE mobilization. The role of TAG as a source for fatty acids in cerulenin treated cells has now to be shown by testing TAG lipase mutants.

Another interesting finding was that STE hydrolase mutants showed resistance to the antifungal compound nystatin. The polyene drug nystatin preferentially binds to ergosterol in membranes. Thus, altered sensitivity to this drug often indicates that the structure of the plasma membrane is disturbed due to altered ergosterol concentrations. The fact that already a *yeh1Δ*, *yeh2Δ*, or *tgl1Δ* single-mutant shows resistance to nystatin could indicate that any of the three STE hydrolases is important for providing sufficient ergosterol for the plasma membrane and that every one of these lipases is thus important for sterol homeostasis. The altered sensitivity to nystatin suggests that STE hydrolase mutants have altered levels of sterols in their plasma membrane. To confirm this hypothesis additional experiments, such as an analysis of the lipid composition of the plasma membrane of STE hydrolase mutants, are necessary. Another approach to determine the physiological role of STE hydrolases in yeast was to examine the ability of mutants to restart growth from stationary phase. Since STE accumulate in the stationary phase and STE mobilization could be important for rapid

initiation of growth, we tested if a strain, unable to mobilize STE, is able to restart growth from stationary phase. Our results showed that a STE hydrolase triple-mutant seems to be able to start growth when kept for short time under nutrient-deprived conditions, although one cannot exclude that the cells are not fully depleted for nutrients at this stage. However, longer incubation of stationary phase triple-mutant cells in water caused loss of viability as determined by methylene blue staining. This loss of viability of the mutant seems to be independent of the deficiency to recycle free sterols from STE for rapid growth initiation. More likely and as already indicated by resistance to nystatin, the *yeh1Δ yeh2Δ tgl1Δ* mutant could "suffer" from serious perturbations of the plasma membrane which would consequently cause death of the cells when kept in water for some time. If the triple-mutant would only have a defect for growth initiation when transferred to fresh media, one could expect that independent of this CFU defect the cells remain viable in water. The results from these two experiments point to an important role of STE hydrolases in cellular sterol homeostasis to ensure sufficient levels of ergosterol for bulk membrane functions. Of course it cannot be ruled out that the STE pool is needed as the source for free sterols to restart the growth from stationary phase and that STE hydrolases are necessary for the ability to use those stored lipids, but the lipase triple-mutant turned out to be none-viable under this experimental condition, and so this hypothesis could not be confirmed.

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Additional experiments Part II:
Membrane topology and subcellular localization of Yeh2p

1. Introduction

We showed recently that one of the STE hydrolases in yeast, namely Yeh2p, is localized to the plasma membrane / cell periphery (1). This was an unexpected finding because such lipases were thought to be found closely associated with their substrate, which are STE. Neutral lipids, including STE, are exclusively stored in intracellular lipid particles which are found primarily adjacent to the vacuole (2). Yeh1p and Tgl1p, two of three yeast STE hydrolases, are associated with lipid particles, but as mentioned above, the third lipase, Yeh2p, is localized to the plasma membrane / cell periphery and seems to be absent from lipid particles (1, 3). Interestingly, all three lipases have predicted transmembrane domains (TMD) in their sequences and solubilization experiments showed that they indeed behave as integral membrane proteins. To understand the action of these lipases on a more mechanistic level we examined their membrane topology in more detail by analyzing the accessibility of C- and N-terminal tags to cleavage by proteinase K. This analysis showed that the C-terminus with the active site of Yeh2p is in a luminal/intracellular compartment, which was not defined further (1). The following approaches should help to define the subcellular compartment as well as the membrane topology of the STE hydrolase Yeh2p more precisely.

2. Materials and Methods

2.1. Strains, media, and growth conditions

Yeast strains used in this study are listed in Table 1. Strains were cultivated in YPD rich media (1% Bacto yeast extract, 2% Bacto peptone (USBiological Swampscott, MA), 2% glucose) or YPGal (1% Bacto yeast extract, 2% Bacto peptone (USBiological Swampscott, MA), 2% galactose) at 24°C or 30°C. N- and C-terminal GFP tagging of *YEH2* was performed by homologous recombination with the PCR fusion cassette from pFA6a-HIS3MX6-PGAL1-GFP(S65T) and pFA6a-GFP(S65T)-HIS3MX6 (4). PCR reaction was performed as described before, the resulting PCR fragments were transformed into wild-type strain BY4742, and correct integration of the fusion cassette was confirmed by colony PCR (1).

2.2. Proteinase K treatment of intact cells

Proteinase K treatment of intact cells was performed essentially as described (8) with minor modifications. Fresh overnight cultures were diluted in 10 ml YPD or YPGal and further grown until an OD_{600} of ~1. Next, equivalents of 4 OD_{600} units were harvested and washed once with buffer A (10 mM Tris-HCl pH 7.5, 1 mM EDTA, 20 mM NaF, 20 mM NaN_3). Cells were then resuspended in 2 ml buffer A with 10 mM DTT and incubated for 10 min at 30°C in a water bath. Cells were centrifuged, resuspended in 1 ml buffer B (600 mM sorbitol, 10 mM K-Pi pH 7.5, 20 mM NaF, 20 mM NaN_3) and 3 units Quantazyme™ per OD_{600} were added. The cell wall was digested during incubation for 30 min at 30°C and spheroblasts were carefully pelleted by centrifugation and resuspended in 1 ml buffer A with 600 mM sorbitol. Next, spheroblasts were divided into two aliquots (2 OD_{600} each) and incubated with and without 200 µg/ml proteinase K (SIGMA) for 30 min at 30°C. The reaction was stopped by the addition of PMSF to a final concentration of 2 mM and incubation on ice for 5 min. Proteins were then extracted essentially as already described (5) and proteins equivalent to 0.5 OD_{600} cells subjected to 8% SDS-PAGE and western blot analysis.

Table 1: *Saccharomyces cerevisiae* strains used in this study

Strain	Relevant genotype	Source or reference
YRS2086	*MATα his3Δ1 leu2Δ0 ura30 lys2Δ0 YEH2-GFP-HIS3MX6*	This study; 1
YRS2083	*MATα his3Δ1 leu2Δ0 ura30 lys2Δ0 HIS3MX6-GAL1-GFP-YEH2*	This study; 1

2.3. Isolation of PAMs and plasma membrane

PAM and plasma membrane was isolated as described earlier (6). In brief, crude plasma membrane was isolated from a culture volume of 2 litres grown to high OD_{600}. The resulting membrane pellet was resuspended in 5 mM Mes, 0.2 mM EDTA, pH 6.0, with 10 strokes in a loose-fitting Dounce homogenizer and layered on top of a sucrose density gradient made of 10 ml 38% (w/w), 10 ml 43% (w/w), and 10 ml 53% (w/w) sucrose in 5 mM Mes, 0.2 mM EDTA, pH 6.0. Centrifugation was carried out at 100,000 x g for 2.5 h in an AH627 swing-out rotor (Beckman). The PAM fraction was collected from the top of the gradient, diluted 3-fold with 10 mM Tris-HCl, pH 7.5, sedimented at 100,000 x g for 45 min using a T865 rotor (Sorvall) and finally resuspended in 500 µl 10 mM Tris-HCl, pH 7.5 using a loose-fitting Dounce homogenizer. The highly purified plasma membrane was withdrawn from the

43%/53% sucrose interface, the suspension was diluted 3-fold with 10 mM Tris-HCl, pH 7.5, and membranes were pelleted by centrifugation at 48,000 x g for 20 min in a SS-34 rotor (Sorvall) and resuspended in 500 µl 10 mM Tris-HCl, pH 7.5 by 5 strokes with a loose-fitting Dounce homogenizer. Both, plasma membrane and PAM were stored at -70°C.

2.4. Western blot analysis

Protein concentrations were determined by the method of Lowry et al. (7), with the Folin reagent and bovine serum albumin as the standard. Proteins were precipitated with 10% trichloroacetic acid (TCA), resuspended in sample buffer, and separated by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis. Western blots were probed with rabbit antisera against GFP (1:5,000; Torrey Pines Biolabs, Inc., Houston, Tex.), Sec61 (1:1,000), Pma1 (1:50,000), and Gas1 (1:5,000, a kind gift from A. Conzelmann, University of Fribourg).

3. Results

3.1. Proteinase K treatment of intact cells indicates that the C-terminus of Yeh2p is not facing the extracellular space

The membrane topology prediction for Yeh2p, as determined earlier, suggested that the C-terminus of the lipase faces the extracellular site of the plasma membrane (1). However, accessibility to cleavage by protease of N- and C-terminal GFP-tagged Yeh2p was determined by using microsomes generated from isolated plasma membrane. Although the C-terminus of Yeh2p was protease protected in this experimental approach, as was the cell surface marker Gas1p, it does not necessarily mean that this part of the lipase indeed faces the cell surface. In fact, during isolation of plasma membrane associated membranes like ER membranes are also co-purified. In other words, isolating plasma membrane for protease protection assays leads also to the enrichment of microsomes from co-purified membranes in which the protein of interest may primarily reside. Furthermore, to validate the results from such a protease protection assay it has to be shown that the microsomes stay intact during the time of the experiment. For this, a marker protein is chosen which is found at either side of the plasma membrane. For our purpose we have chosen Gas1p as a marker protein which is a GPI-anchored glycoprotein exposed to the cell surface of yeast (8). Protease treatment of microsomes from isolated plasma membrane showed that Gas1p is protected from degradation, which indicates that the microsomes are closed “inside out” vesicles. The results from our experiments indicate that, because the C-terminus of Yeh2p is also protease

protected, as is Gas1p, it faces the extracellular side. To confirm this membrane topology of the lipase, we tested again the accessibility of C- and N-terminal parts of Yeh2p, but this time in intact cells. In this assay the cell surface protein Gas1p is rapidly degraded by proteinase K, whereas the C-terminal part of Yeh2p was not accessible to degradation by the protease (Fig.1A). This result together with the result from the topology assessment performed before, leads to the conclusion that the C-terminus Yeh2p resides in a luminal compartment at the plasma membrane but does not faces the extracellular space (Fig.1B).

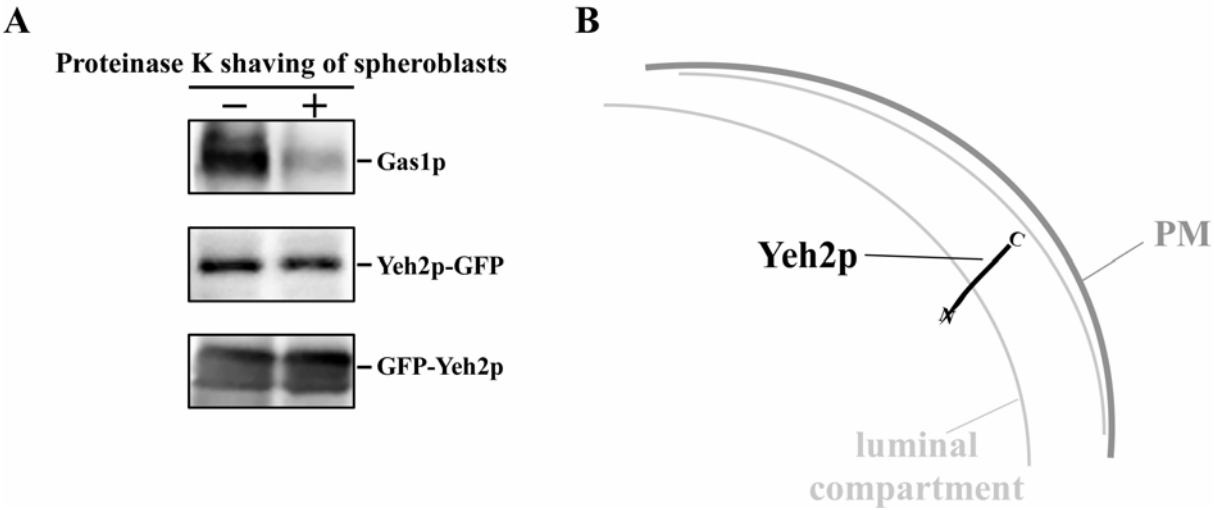

Figure 1: The C-terminal part of Yeh2p is not facing the extracellular space. (A) Proteinase K shaving of spheroblast. Cells expressing GFP-tagged Yeh2p (Yeh2p-GFP, YRS2086; GFP-Yeh2p, YRS2083) were grown to OD_{600} of ~1. The cell wall was removed and intact spheroblasts were treated with or without 200 µg/ml proteinase K at 30°C for 30 min. After the reaction was stopped, spheroblasts were broken, proteins precipitated with 10% TCA, and proteins equivalent to 0.5 OD_{600} were separated by electrophoresis and analysed by western blot for the presence of the GFP-epitope and Gas1p. (B) Model of localization and topology of Yeh2p in a luminal compartment at the plasma membrane (PM).

3.2. Subcellular localization of Yeh2p – Isolation of PAM

The finding that Yeh2p does not faces the extracellular side but is rather localized to a luminal compartment at the cytoplasmatic side of the plasma membrane made us start to search for this compartment. Daum and co-workers identified and isolated a subfraction of the ER which is closely associated with the plasma membrane, called plasma membrane associated membrane (PAM) (6). Interestingly, several lipid synthesizing enzymes are highly enriched in PAM, among them Erg9p, Erg1p, and Erg6p of the ergosterol biosynthetic pathway. Thus it is not surprising that PAM has a high capacity so synthesize certain sterols as well as other lipids. This interesting “new compartment” attracted our attention as a possible place where

Yeh2p may reside. To show if the STE hydrolase is indeed enriched in this subfraction of the ER, we isolated plasma membrane and PAM from a strain expressing Yeh2p-GFP and performed western blot analysis. In Table 2 the relative enrichment of Yeh2p-GFP and the marker proteins in the different fractions is shown. The STE hydrolase Yeh2p is highly enriched in the crude plasma membrane (PM and PAM together) and also in the isolated PAM as compared to the homogenate (Fig.2). In contrast to this, the enrichment of Yeh2p-GFP in the purified plasma membrane fraction (PM without PAM) is only half of that found in the PAM fraction. As a control for the purity of the PAM isolation the distribution of Sec61p, an ER marker protein, was also determined. As can be seen, Sec61p is detectable in the crude plasma membrane fraction, nearly absent from the purified plasma membrane but ~7-fold enriched in the PAM fraction (Fig.2). This is consistent with the view that PAMs are a subfraction of the ER. In other words, the absence of Sec61p in the purified plasma membrane and the high amounts of the marker protein in the PAM fraction confirms that the PAM isolation was successful. However, the relative amounts of yeast plasma membrane ATPase, Pma1p, seem to be distributed equally between the fractions, although it should be depleted from PAM (Fig.2). Taken together, we could show that Yeh2p-GFP is enriched in PAM but also found it in the purified plasma membrane which suggests that the lipase is not localized exclusively in PAM.

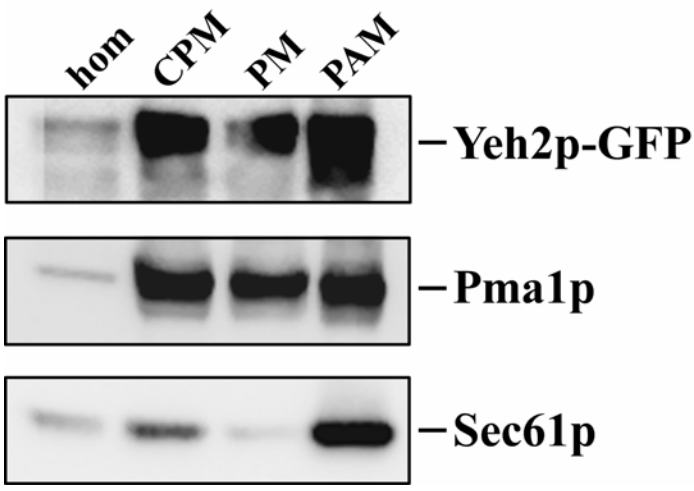

Figure 2: Yeh2p-GFP is enriched in PAM. Plasma membrane was isolated from a wild-type strain expressing Yeh2p-GFP (YRS2086) as described in Materials and Methods. Adjusting the pH to 6.0 resulted in the dissociation of PAM from plasma membrane. PAM was then separated from highly purified plasma membrane by sucrose density centrifugation. Proteins of each fraction were precipitated by TCA, and 5 µg proteins per lane were separated by electrophoresis and probed for the GFP-epitope and for the marker proteins Sec61 and Pma1. (hom, homogenate; CPM, crude plasma membrane; PM, purified plasma membrane; PAM, plasma membrane associated membrane;)

Table 2: Signal intensity of the immunoreactive band in the homogenate was set to 1, and corresponding intensities in CPM, PM and PAM were calculated.

	Relative enrichment (-fold)		
	plasma membrane crude (CPM)	plasma membrane purified (PM)	PAM
Yeh2p-GFP	8.95	4	8.93
Pma1p	14.3	11.4	13
Sec61p	2.7	0.5	7.4

4. Discussion

The studies presented here provide additional information about the topology and the subcellular localization of the yeast STE hydrolase, encoded by *YEH2*. We recently showed that Yeh2p is localised to the plasma membrane in yeast (1). This finding was somewhat surprising, as such a lipase was expected to be associated with lipid particles, the storage organelle for STE. In fact, two other yeast STE hydrolases, namely Yeh1p and Tgl1p, are associated with lipid particles, where STE mobilization is likely to take place. Therefore, it is unclear why the third STE hydrolase Yeh2p is found at the plasma membrane. Interestingly, all three STE hydrolases behave as integral membrane proteins and a membrane topology model of Yeh2p suggested that the active site of this lipase projects to the extracellular side of the plasma membrane. To verify this membrane topology, we tested the accessibility of the C- and N-terminal regions of Yeh2p on the cell surface by protease treatment of intact cells. This experimental approach clearly showed that the C-terminal part with the active site of the protein is not degraded by protease and thus is not facing towards the extracellular space. Moreover, this part of the lipase is rather in a luminal compartment at the plasma membrane, which could be peripheral ER or PAM. This of course then raised the question in which compartment at the plasma membrane Yeh2p resides. Subcellular fraction then revealed that Yeh2p is enriched ~8.9-fold in PAM, although not exclusively. Yeh2p was also found to be enriched in the purified plasma membrane which should be devoid of PAM or peripheral ER. Several sterol-synthesizing enzymes are enriched in PAM, and together with the sterol synthesizing capacity of this compartment, PAM may contribute to sterol homeostasis (6). It would thus not be surprising to also find a STE hydrolase in this compartment. The yeast STE hydrolase Yeh2p could release sterol precursors from STE, e.g. zymosterol, and thus provide substrate for the sterol-synthesizing enzymes in PAM. Apart from this function, one could also imagine that Yeh2p hydrolyzes STE to directly supply the need of free ergosterol in the plasma membrane. In this scenario the sterol liberated from the hydrolysis reaction has not to

be transported to the plasma membrane but instead is already at the place where it is needed. This would also allow rapid supply of ergosterol from the STE pool for growing membranes. It has not been verified until now that STE mobilization happens exclusively at lipid particles and it is thus possible that lipid particles containing STE migrate to the plasma membrane and that Yeh2p hydrolyzes certain STE there.

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Additional experiments Part III:

Blue Native gel electrophoresis of Yeh1p, Yeh2p, and Tgl1p

1. Introduction

Fully active forms of lipases often consist of protein complexes with associated protein factors, so called co-lipases. Pancreatic lipase (HGL) for example needs an additional co-lipase which facilitates the lipolytic activity of the enzyme. The co-factor enables HGL to anchor to the water-lipid interface (1). In addition, lipases often form catalytically active dimers like lipoprotein lipase (LPL), which plays a central role in the metabolism of blood lipids (2). To determine if the three STE hydrolases of yeast, namely Yeh1p, Yeh2p, and Tgl1p, form dimers within the cells or eventually have co-factors (co-lipases) associated with them, blue native electrophoresis of membrane extracts was performed. Blue native gel electrophoresis was originally developed for the separation of membrane proteins and multiprotein complexes of great mass, as such protein complexes are not disrupted by mild detergents (3). In this method, mild detergents are used for protein solubilization and Coomassie Blue dye is added to charge the proteins negatively. As a consequence, all proteins migrate to the anode. Native protein complexes thus stay intact and are size-separated through the gel. By comparing the molecular mass of the protein in the native gel to the one in a SDS-PAGE gel it can be judged whether the protein is in a complex or migrates as a monomer.

2. Materials and methods

2.1. Strains, media, and growth conditions

Yeast strains used in this study are listed in Table 1. Strains were cultivated in YPD rich media (1% Bacto yeast extract, 2% Bacto peptone (USBiological Swampscott, MA), 2% glucose) and grown at 24°C to OD_{600} of ~1.

Table 1: *Saccharomyces cerevisiae* strains used in this study

Strain	Relevant genotype	Source or reference
YRS1974	*MATα his3Δ1 leu2Δ0 ura30 met15Δ0 YEH11-GFP-HIS3MX6*	This study; 5
YRS1858	*MATα his3Δ1 leu2Δ0 ura30 met15Δ0 TGL1-GFP-HIS3MX6*	This study; 5
YRS2086	*MATα his3Δ1 leu2Δ0 ura30 lys2Δ0 YEH2-GFP-HIS3MX6*	This study; 5

2.2. Preparation of Microsomal Membranes and Solubilization of Membrane Proteins

Microsomal membranes were prepared as follows. Cells equivalent to 300 OD_{600} units were collected by centrifugation (3,000 x *g*; 10 min), resuspended in 100 mM Tris (pH 9.4), 10 mM dithiothreitol, and incubated for 10 min at 30°C. Cells were pelleted (3,000 x *g*; 5 min) and resuspended in 0.2x YPD medium, 0.6 M sorbitol, 10 mM KPi, pH 7.5. Cells were converted to spheroplasts by incubation with 16 U of Zymolyase (Seikagaku Corporation, Tokyo, Japan) for 20 min at 30°C. Spheroplasts were pelleted (4,000 x *g*, 20 min), resuspended in lysis buffer A (0.2 M sorbitol, 50 mM potassium acetate, 20 mM HEPES pH 6.8, 2 mM EDTA, 0.2 mM phenylmethylsulfonyl fluoride [PMSF] supplemented with a protease inhibitor cocktail [Complete; Roche-Diagnostics, Rotkreuz, Switzerland]), and lysed with a Dounce homogenizer. The homogenate was then centrifuged at 13,000 x g for 30 min in a SS34 rotor (Sorvall) at 4°C to obtain the microsomal membrane pellet (P13) which was resuspended in 10 mM Tris pH 7.5, 15% glycerol and frozen at -80°C. Protein concentration was determined by the method of Lowry et al. (4), with the Folin reagent and bovine serum albumin as standard. Proteins equivalent to 25 µg were resuspended in solubilization buffer (50 mM NaCl, 5 mM aminocaproic acid, 1 mM EDTA, 50 mM imidazole) to a final concentration of 1 µg/µl and incubate 5 min on ice. The solubilization of membrane proteins (25 µg each) was achieved by adding digitonin to a final detergent to protein ratio of either 4 or 8. After incubation on ice for 30 min, unsolubilized material was removed by centrifugation at 16,000 x g for 20 min in an Eppendorf centrifuge. The supernatant was removed and glycerol was added to a final concentration of 10 %. As a control to obtain the monomeric, denaturated, forms of proteins, the same procedure was carried out using SDS instead of digitonin which was adjusted at a concentration of 0.5 %. The solubilized samples were immediately used for Blue native gel electrophoresis.

2.3. Blue Native PAGE and Western blot analysis

Buffers and gel composition were used as previously described (3). Coomassie Blue G250 in 500 mM aminocaproic acid was added to the samples (25 µl solubilized proteins) to a final detergent to dye ratio of 4 to 1 and the sample was loaded onto 4-10% or 6-18% polyacrylamide gradient gels. Electrophoresis was carried out at 4°C with 10 mA. After one third of the running time the deep blue cathode buffer with 0.02 % Coomassie Blue was removed and replaced by a slightly blue cathode buffer (0.002 % Coomassie Blue), and the run was continued with 20 mA. The following electrophoretic transfer to PVDF membrane (Immobilon-P, Millipore) was carried out for 30 min with 100 mA at 4°C. GFP-tagged lipases

were identified by probing with rabbit antisera against GFP (1:5,000; Torrey Pines Biolabs, Inc., Houston, Tex.) and detection with enhanced chemiluminescence (SuperSignal West Pico; Pierce Biotechnology, Inc.).

3. Results

To examine a possible interaction of the STE hydrolases Yeh1p, Yeh2p, and Tgl1p with co-factors, and to determine whether the lipases form dimers under physiological conditions, the molecular weight of native lipases was analysed by blue native gel electrophoresis. This method allows the preservation of the tertiary and quaternary structures of native protein complexes and their separation according to their molecular weight (3). Cells expressing C-terminally GFP-tagged lipases were grown on rich media to logarithmic phase, and microsomal proteins were solubilized in digitonin and analyzed by blue native gel electrophoresis. As shown in Fig.1A, GFP-tagged lipases reacting with the anti-GFP antibody are found as sharp bands at a molecular weight of ~66kDa. The migration of all three STE hydrolases Yeh1p, Yeh2p, and Tgl1p in the supposed native form under physiological conditions is the same as for the SDS-denaturated lipases (lane: SDS). This indicates that the lipases are not in a protein complex with other proteins and/or with themselves. To confirm this, the extracts were separated again on a higher percentage gradient gel. As can be seen in Fig.1B, separation of the protein extracts on a 6% - 18% gradient gel led to the same result. There is no relevant difference in the molecular mass of the lipases in their native form compared to the SDS-denaturated form, strongly indicating that the lipases are not in protein complexes.

4. Discussion

The purpose of this experiment was to determine if the STE hydrolases of yeast, namely Yeh1p, Yeh2p, and Tgl1p, form dimers and/or are associated with protein partners under physiological conditions. Recently, it has been shown that human LPL forms enzymatically active dimers, and activity of another human lipase, HGL, is dependent on a co-lipase which enables HGL to anchor on lipid / water interfaces (2, 1). To investigate whether this is also true for STE hydrolases of yeast, we performed blue native gel electrophoresis to resolve possible protein complexes. The result of the blue native electrophoresis showed that all three STE hydrolases migrate the same distance in agreement with the molecular sizes of their monomeric forms, even when isolated under conditions which do not disrupt protein complexes. Taken together, the experiment indicates that Yeh1p, Yeh2p, and Tgl1p (i) do not

form dimers and (ii) seem not to have any co-factors associated under physiological conditions.

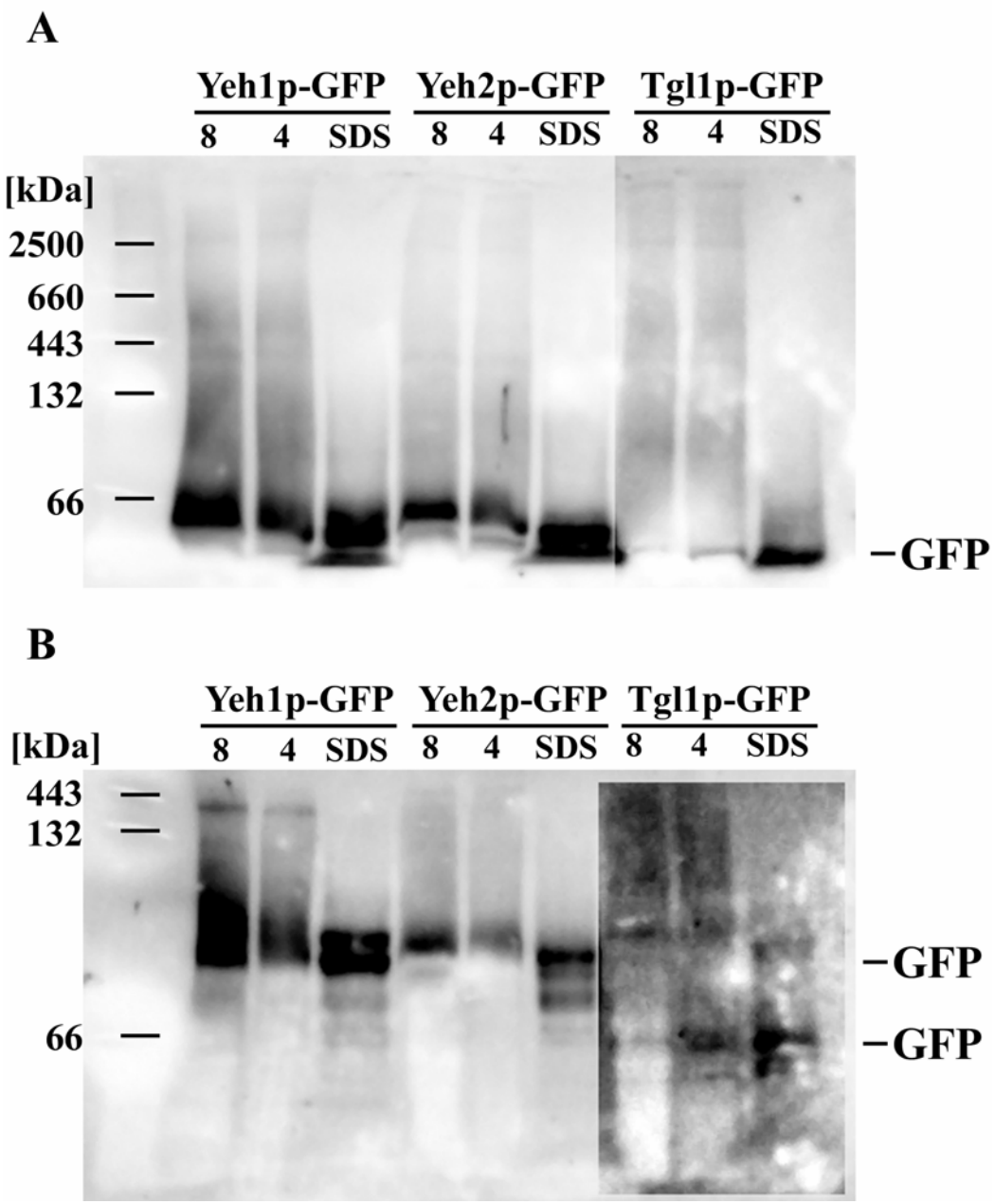

Figure 1: Blue native gel electrophoresis of STE hydrolases. Cells expressing C-terminally GFP-tagged lipases (Yeh1p-GFP, YRS1974; Yeh2p-GFP, YRS2086; Tgl1p-GFP, YRS1858) were grown to OD_{600} of ~1. Cells were harvested, microsomes were prepared, and membrane proteins were solubilized in digitonin or SDS and processed for blue native PAGE as described in Materials and Methods. Solubilized proteins (25 µg per lane) were loaded on a (A) 4% - 10% or (B) 6% - 18% polyacrylamide gradient gel and separated at 4°C. Proteins were transferred onto a PVDF membrane and probed with antibodies against GFP. Marker proteins used were bovine serum albumin (monomeric and dimeric form, 66 and 132 kDa), apoferritin (443 kDa), thyroglobulin (660 kDa), and alpha-ketoglutarate dehydrogenase (2500kDa).

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CHAPTER III

YEH1 constitutes the major steryl ester hydrolase under heme-deficient conditions

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Yeh1 Constitutes the Major Steryl Ester Hydrolase under Heme-Deficient Conditions in *Saccharomyces cerevisiae*

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Steryl esters are stored in intracellular lipid droplets from which they are mobilized upon demand and hydrolyzed to yield free sterols and fatty acids. The mechanisms that control steryl ester mobilization are not well understood. We have previously identified a family of three lipases of *Saccharomyces cerevisiae* that are required for efficient steryl ester hydrolysis, Yeh1, Yeh2, and Tgl1 (R. Köffel, R. Tiwari, L. Falquet, and R. Schneiter, Mol. Cell. Biol. 25:1655–1668, 2005). Both Yeh1 and Tgl1 localize to lipid droplets, whereas Yeh2 is localized to the plasma membrane. To characterize the precise function of these three partially redundant lipases, we examined steryl ester mobilization under heme-deficient conditions. *S. cerevisiae* is a facultative anaerobic organism that becomes auxotrophic for sterols and unsaturated fatty acids in the absence of molecular oxygen. Anaerobic conditions can be mimicked in cells that are deficient for heme synthesis. We here report that Yeh1 is the sole active steryl ester hydrolase under such heme-deficient conditions, indicating that Yeh1 is activated whereas Yeh2 and Tgl1 are inactivated by the lack of heme. The heme-dependent activation of Yeh1 is mediated at least in part by an increase in steady-state levels of Yeh1 at the expense of Yeh2 and Tgl1 in exponentially growing cells. This increase in steady-state levels of Yeh1 requires Rox3, a component of the mediator complex that regulates transcription by RNA polymerase II. These data thus provide the first link between fat degradation and the transcriptional control of lipase activity in yeast.

Sterols are essential lipids of eukaryotic cells, where they occur in two major forms: free sterols and steryl esters. Free sterols are synthesized in the endoplasmic reticulum (ER) membrane, and they are greatly enriched at the plasma membrane, which harbors 90% of the free sterol pool of a cell (13). Steryl esters, on the other hand, serve to store fatty acids and sterols for energy production and membrane synthesis. These nonmembrane-forming neutral lipids are deposited in intracellular lipid droplets. The conversion of free sterols and acyl coenzyme A's to steryl esters is catalyzed by acyl coenzyme A:sterol acyltransferases (ACATs) that are localized in the ER membrane (3). The formation and hydrolysis of steryl esters are important in maintaining sterol homeostasis, as the steryl ester pool conceptually serves to buffer both excess and a lack of free sterols (2).

Saccharomyces cerevisiae harbors two ACAT genes, *ARE1* and *ARE2*. Deletion of both genes results in the absence of steryl esters but does not compromise mitotic growth, indicating that synthesis of steryl esters is not essential under standard growth conditions (27, 28). Are2 provides the major activity to esterify the mature sterol of yeast, ergosterol, under aerobic conditions, and its activity is important to complete meiosis, as an *are2*Δ homozygous diploid has a reduced sporulation efficiency and arrests after the first meiotic division (28). Are1, on the other hand, preferentially esterifies sterol intermediates and is upregulated under heme-deficient conditions (10, 24, 25, 30). These observations indicate that Are1 is physiologically important under anaerobic conditions, when heme is limiting and sterol precursors accumulate.

Steryl esters synthesized by Are1 and Are2 are stored in intracellular lipid droplets from which they can be remobilized upon demand and are then hydrolyzed by the action of three steryl ester hydrolases, Yeh1, Yeh2, and Tgl1 (8, 11, 14, 17). Yeh1 and Tgl1 localize to lipid particles, whereas Yeh2 is enriched at the plasma membrane (8, 11, 17). All three lipases are membrane anchored, and a triple mutant strain lacking all three lipases lacks steryl ester hydrolysis in vivo, indicating that together Yeh1, Yeh2, and Tgl1 account for all the steryl ester hydrolase activity that is present in yeast (11). The fact that the lipase triple mutant is viable furthermore indicates that steryl ester hydrolysis is not essential for mitotic growth (11).

The aim of this study was to characterize the role of the three lipases in steryl ester hydrolysis in more detail and to begin to dissect the apparent functional redundancy of these lipases. We here concentrate on the role of the three lipases under heme-deficient conditions and show that under these conditions, Yeh2 and Tgl1 are inactive in vivo and that all the steryl ester hydrolase activity present in heme-deficient cells is attributed to Yeh1. Activation of Yeh1 under heme deficiency is accompanied by an upregulation of steady-state levels of Yeh1 at the expense of Tgl1 and Yeh2 in exponentially growing cells. The heme-dependent inactivation of Yeh2 and Tgl1 is overcome by overexpression of the enzymes, indicating that expression of Yeh2 and Tgl1 is rate limiting under heme deficiency. Activation of Yeh1 under these conditions requires Rox3, a component of the mediator complex that controls RNA polymerase II activity, indicating that steryl ester degradation is controlled at least in part by transcriptional regulation of *YEH1* expression (6, 18).

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MATERIALS AND METHODS

Yeast strains and growth conditions. Yeast strains used in this study are listed in Table 1. Strains bearing single deletions of nonessential genes were obtained from EUROSCARF (see http://www.rz.uni-frankfurt.de/FB/fb16/mikro/euroscarf/index.html [26]). Strains were cultivated in YPD-rich media (1% Bacto yeast extract, 2% Bacto peptone [USBiological, Swampscott, MA], 2% dextrose) or minimal media. Media supplemented with sterols and fatty acids contained 5 mg/ml Tween 80 and 20 μg/ml ergosterol, cholesterol, or lanosterol (Sigma Chemical Co., St Louis, MO). *hem1*Δ mutant cells were supplemented with 20 μg/ml delta-aminolevulinic acid (ALA). Selection for the *kanMX4* marker was on media containing 200 μg/ml G418 (Gibco-BRL, Life Technologies). Double and triple mutant strains were generated by crossing of single mutants and by gene disruption, using the PCR deletion cassettes (15) and the gene-specific primers used for the initial characterization of the three lipases (11). The plasmid pHEM1-LEU2 containing the *hem1*::*LEU2* disruption cassette (kindly provided by I. Hapala, Slovak Academy of Sciences, Bratislava, Slovak Republic) was cut with BamHI/HindIII to release the disruption cassette, and yeast transformants were selected on minimal media without leucine but supplemented with ALA. Correct insertion of the disruption cassette at the *HEM1* locus was confirmed by phenotypic analysis of the transformants, i.e., growth on ALA-supplemented media but no growth on nonsupplemented media. Yeast was transformed by treatment with lithium acetate (7).

Western blot analysis. Protein concentrations were determined by the method of Lowry et al. (16), using the Folin reagent and bovine serum albumin as standards. Proteins were precipitated with 10% trichloroacetic acid, resuspended in sample buffer, and separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Western blots were probed with rabbit antisera against green fluorescent protein (GFP) (1:5,000; Torrey Pines Biolabs, Inc., Houston, TX), Kar2 (1:5,000; M. Rose, Princeton University, New Jersey), or Wbp1 (1:1,000; M. Aebi, ETH Zurich, Switzerland).

Fluorescence microscopy. In vivo localization of GFP-tagged versions of Yeh1, Yeh2, and Tgl1 in the heme-deficient background was performed by fluorescence microscopy, using a Zeiss Axioplan 2 (Carl Zeiss, Oberkochen, Germany) equipped with an AxioCam CCD camera and AxioVision 3.1 software.

In vivo neutral lipid mobilization. The steryl ester pool was labeled by incubating the cells either with 0.025 μCi/ml [^{14}C]cholesterol or with 10 μCi/ml [^{3}H]palmitic acid (American Radiolabeled Chemicals Inc., St. Louis, MO) for 16 h at 24°C. Cells were then washed and diluted into liquid YPD media containing Tween 80 and either cholesterol or ergosterol and 30 μg/ml terbinafine to block squalene epoxidase (9). The inclusion of terbinafine is not required to induce the mobilization of steryl esters but prevents the formation of lanosterol and thus more closely mimics an anaerobic block in sterol biosynthesis than it does heme deficiency alone. Aliquots of cells were removed at the time points indicated. Cells were frozen and broken with glass beads, and lipids were extracted with chloroform-methanol (1:1 [vol/vol]). Radioactivity in the lipid extract was determined by scintillation counting, and equal counts were dried. Lipids were separated on thin-layer chromatography (TLC) plates (Merck, Darmstadt, Germany) developed in petroleum ether-diethylether-acetic acid (70:30:2 [by volume]) and quantified by scanning with a Berthold Tracemaster 40 automatic TLC linear analyzer. TLC plates were then exposed to a tritium-sensitive screen and visualized using a PhosphorImager (Bio-Rad Laboratories, Hercules, CA).

Overexpression of steryl ester hydrolases and quantification of the endogenous steryl ester pool. Heme-deficient strains were cultivated in cholesterol-containing media with either glucose (repressing conditions) or galactose (inducing conditions) as the carbon source. The neutral lipid pool was labeled to steady-state levels by incubating the cells with 0.025 μCi/ml [^{14}C]cholesterol for 16 h at 30°C. Cells were diluted into YPD or YP-galactose liquid media containing terbinafine (30 μg/ml). Aliquots of cells were removed after 6 h of growth, and 3 optical density (OD) units were used for Western blot analysis. The remaining cells (~20 OD units) were washed and broken, and lipids were extracted as described above. Equal counts were dried, and lipids were separated on TLC plates developed in petroleum ether-diethylether-acetic acid (70:30:2 [by volume]) and quantified by scanning with a Berthold Tracemaster 40 automatic TLC linear analyzer.

Northern analysis. Total RNA was prepared by extraction of cells with hot acidic phenol. RNA (20 μg) was separated on denaturing 1% agarose gels, transferred to nitrocellulose membranes (NEN GeneScreen Plus), and hybridized overnight with ^{32}P-labeled RNA probes made by random priming of gene-specific PCR products. Membranes were washed at high stringency and exposed to a PhosphorImager screen.

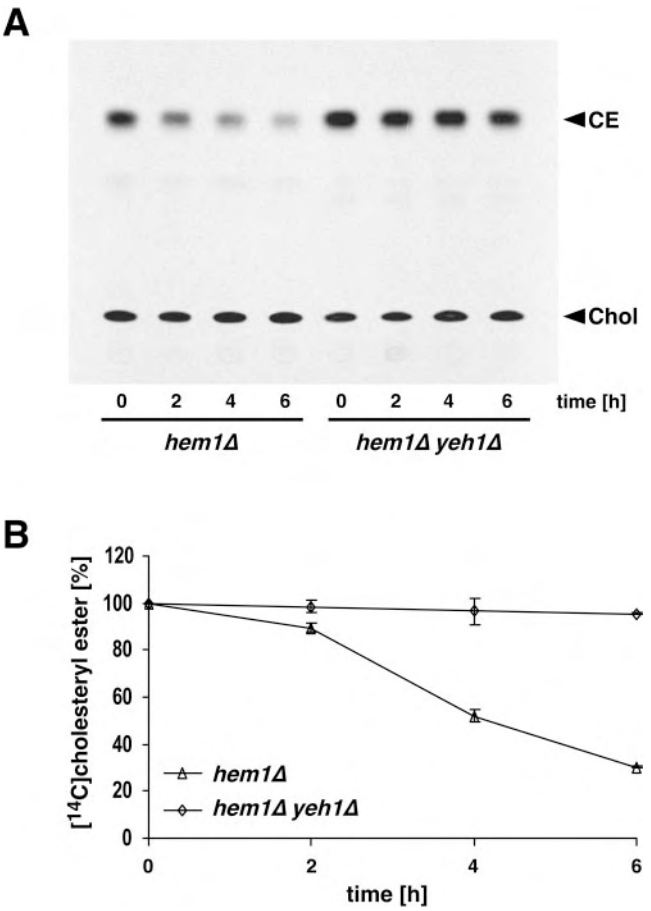

FIG. 1. *YEH1* is required for steryl ester mobilization in a heme-deficient background. (A) Heme-deficient wild-type (YRS1707) and *yeh1*Δ mutant (YRS1710) cells were labeled for 16 h with [^{14}C]cholesterol, and the kinetics of steryl ester mobilization in vivo was analyzed by determining steryl ester levels at 0, 2, 4, and 6 h after the dilution of cells into fresh media. Lipids were extracted and analyzed by TLC, as described in Materials and Methods. Chol, cholesterol; CE, cholesteryl esters. (B) Levels of [^{14}C]cholesteryl esters were quantified by radioscanning of TLC plates and set in relation to the levels at time zero (100%). Values represent means and standard deviations from two independent experiments.

RESULTS

***YEH1* is required for efficient in vivo mobilization of steryl esters in heme-deficient cells.** Given that steryl ester synthesis is differentially regulated by the availability of oxygen, we wondered whether the same may also hold true for steryl ester hydrolysis. Anaerobiosis can be mimicked by heme deficiency, which renders the cells auxotrophic for methionine, sterols, and unsaturated fatty acids, because the synthesis of these compounds require cytochromes and molecular oxygen (5). To examine the rate of steryl ester hydrolysis in heme-deficient steryl ester hydrolase mutants, cells were labeled with [^{14}C]cholesterol and then diluted into fresh media containing cold cholesterol and Tween 80 as a source for unsaturated fatty acids. Samples were then withdrawn after 0, 2, 4, and 6 h of growth, lipids were isolated and separated by TLC, and the level of [^{14}C]cholesterol-labeled steryl esters was quantified by radio scanning of TLC plates. Under these conditions, the

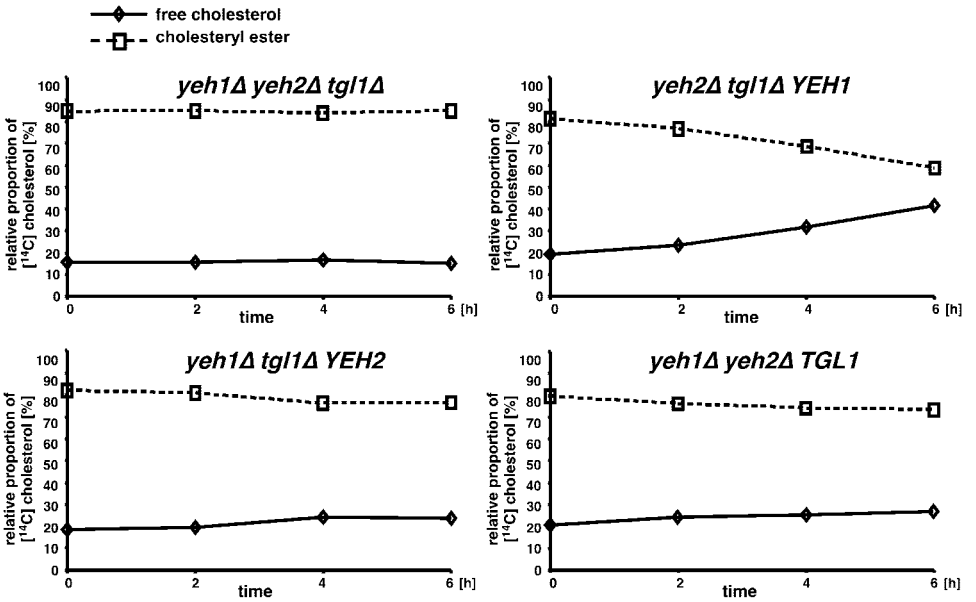

FIG. 2. *YEH2* and *TGL1* do not contribute to steryl ester hydrolysis under heme-deficient conditions. Heme-deficient lipase triple (*yeh1*Δ *yeh2*Δ *tgl1*Δ, YRS1922) and double (*yeh1*Δ *tgl1*Δ, YRS1923; *yeh2*Δ *tgl1*Δ, YRS1961; and *yeh1*Δ *yeh2*Δ, YRS2045) mutant cells were labeled for 16 h with [^{14}C]cholesterol, and the kinetics of steryl ester mobilization in vivo was analyzed after the dilution of cells into fresh media. Lipids were extracted and analyzed by TLC, as described in Materials and Methods. Levels of free and esterified [^{14}C]cholesterol were quantified by radioscanning of TLC plates. Data shown are representative of two independent experiments, with standard deviations of less than 5% between experiments.

heme-deficient wild-type strain efficiently mobilizes steryl esters, resulting in an approximately threefold drop of the steryl ester pool over a 6-h period. Cells lacking *YEH1*, on the other hand, maintained most of their steryl ester pool, indicating that Yeh1 is required for efficient hydrolysis of steryl esters under heme-deficient conditions (Fig. 1). Absence of either *YEH2* or *TGL1*, on the other hand, did not affect the rate of steryl ester mobilization compared to wild-type cells, indicating that Yeh1 may be the only steryl ester hydrolase that is active under heme deficiency (data not shown).

***YEH1* is the only steryl ester hydrolase gene active under heme deficiency.** To examine the contribution of *YEH1*, *YEH2*, or *TGL1* to steryl ester hydrolysis in the absence of the other two hydrolases, we examined steryl ester mobilization in the lipase triple mutant and the three lipase double mutant combinations. Therefore, heme-deficient steryl ester hydrolase triple and double mutant strains were generated and cells were labeled with [^{14}C]cholesterol. The cells were again diluted into fresh media containing cold cholesterol, and the mobilization of the preexisting steryl ester pool was examined over time.

TABLE 1. *S. cerevisiae* strains used in this study

Strain	Relevant genotype	Source and/or reference
YRS1533	*BY4742*; *MAT*α *his3*Δ*1 leu2*Δ*0 ura3*Δ*0 lys2*Δ*0*	EUROSCARF; 26
YRS1707	*BY4742*; *MAT*α *his3*Δ*1 leu2*Δ*0 ura3*Δ*0 lys2*Δ*0 hem1*::*LEU2*	This study
YRS1710	*MAT*α *his3*Δ*1 leu2*Δ*0 ura3*Δ*0 lys2*Δ*0 yeh1*::*kanMX4 hem1*::*LEU2*	This study
YRS1709	*MAT*α *his3*Δ*1 leu2*Δ*0 ura3*Δ*0 lys2*Δ*0 tgl1*::*kanMX4 hem1*::*LEU2*	This study
YRS1708	*MAT*α *his3*Δ*1 leu2*Δ*0 ura3*Δ*0 lys2*Δ*0 yeh2*::*kanMX4 hem1*::*LEU2*	This study
YRS1922	*MAT*? *his3*Δ*1 leu2*Δ*0 ura3*Δ*0 met15*Δ*0 LYS2 yeh1*::*HIS3MX6 yeh2*::*kanMX4 tgl1*::*kanMX4 hem1*::*LEU2*	This study
YRS2045	*MAT*α *his3*Δ*1 leu2*Δ*0 ura3*Δ*0 lys2*Δ*0 yeh1*::*HIS3MX6 yeh2*::*kanMX4 hem1*::*LEU2*	This study
YRS1923	*MAT*α *his3*Δ*1 leu2*Δ*0 ura3*Δ*0 lys2*Δ*0 yeh1*::*HIS3MX6 tgl1*::*kanMX4 hem1*::*LEU2*	This study
YRS1961	*MAT*? *his3*Δ*1 leu2*Δ*0 ura3*Δ*0 met15*Δ*0 LYS2 yeh2*::*kanMX4 tgl1*::*kanMX4 hem1*::*LEU2*	This study
YRS2046	*MAT***a** *his3*Δ*1 leu2*Δ*0 ura3*Δ*0 met15*Δ*0 YEH1-GFP-HIS3MX6 hem1*::*LEU2*	This study
YRS2048	*MAT***a** *his3*Δ*1 leu2*Δ*0 ura3*Δ*0 met15*Δ*0 YEH2-GFP-HIS3MX6 hem1*::*LEU2*	This study
YRS2047	*MAT***a** *his3*Δ*1 leu2*Δ*0 ura3*Δ*0 met15*Δ*0 TGL1-GFP-HIS3MX6 hem1*::*LEU2*	This study
YRS2184	*MAT*? *his3*Δ*1 leu2*Δ*0 ura3*Δ*0 met15*Δ*0 LYS2 yeh2*::*kanMX4 tgl1*::*kanMX4 trp1 TRP1-GAL1-GFP-YEH1 hem1*::*LEU2*	This study
YRS2183	*MAT*α *his3*Δ*1 leu2*Δ*0 ura3*Δ*0 lys2*Δ*0 trp1 yeh1*::*HIS3MX6 tgl1*::*kanMX4 TRP1-GAL1-GFP-YEH2 hem1*::*LEU2*	This study
YRS2182	*MAT*α *his3*Δ*1 leu2*Δ*0 ura3*Δ*0 lys2*Δ*0 trp1 yeh1*::*HIS3MX6 yeh2*::*kanMX4 TRP1-GAL1-GFP-TGL1 hem1*::*LEU2*	This study
YRS1766	*MAT*α *his3*Δ*1 leu2*Δ*0 ura3*Δ*0 lys2*Δ*0 rox3*::*kanMX4 hem1*::*LEU2*	19
YRS2740	*MAT***a** *his3*Δ*1 leu2*Δ*0 ura3*Δ*0 met15*Δ*0 rox3*::*kanMX4 YEH1-GFP-HIS3MX6 hem1*::*LEU2*	This study

This analysis revealed that only cells with a functional *YEH1* were able to mobilize steryl esters, indicating that Yeh1 is the only steryl ester hydrolase that is active under heme-deficient conditions and that in the absence of *YEH1*, neither Yeh2 nor Tgl1 can replace Yeh1 (Fig. 2). In the absence of either Tgl1 or Yeh2 or both lipases, however, Yeh1p-dependent steryl ester hydrolysis is approximately fourfold less efficient that in wild-type cells, indicating that the presence of either Tgl1 or Yeh2 directly or indirectly increases the rate of steryl ester hydrolysis.

Yeh1 is active against different steryl ester substrates. Oxygen-limiting conditions result in the accumulation of intermediates of the sterol biosynthetic pathway, particularly lanosterol, as many of the biosynthetic steps along this pathway are oxygen and/or heme dependent (4, 5, 20). These sterol intermediates are then preferentially esterified by Are1, which provides the major ACAT activity under heme-deficient or anaerobic conditions (10, 24). To examine whether the activation of Yeh1 at the expense of Yeh2 and Tgl1 under heme-deficient conditions is due to a possible substrate preference of the enzyme for nonergosterol-containing steryl esters, we examined the apparent in vivo substrate specificity of Yeh1 towards different steryl esters. Therefore, heme-deficient cells were precultivated in media containing ergosterol, cholesterol, or lanosterol; they were then labeled with [^{3}H]palmitic acid to radiolabel the steryl ester pool and shifted to fresh media containing the type of sterol with which the cells were precultivated, and steryl ester mobilization was analyzed over time. This analysis revealed that the turnover of the different types of sterols remained constant and was in each case dependent on Yeh1, indicating that the activity of Yeh1 under heme deficiency is not due to a substrate preference of the enzyme for nonergosterol-containing steryl esters. In addition, the analysis also revealed that the lack of activity of Yeh2 and Tgl1 under heme deficiency cannot be completely explained by a preference of these enzymes for ergosteryl esters, even though Yeh2 does appear to exhibit increased activity against ergosteryl esters (Fig. 3). Taken together, these observations indicate that the activities of the three lipases are differentially regulated by the heme status of the cells but are largely independent of the type of steryl ester present.

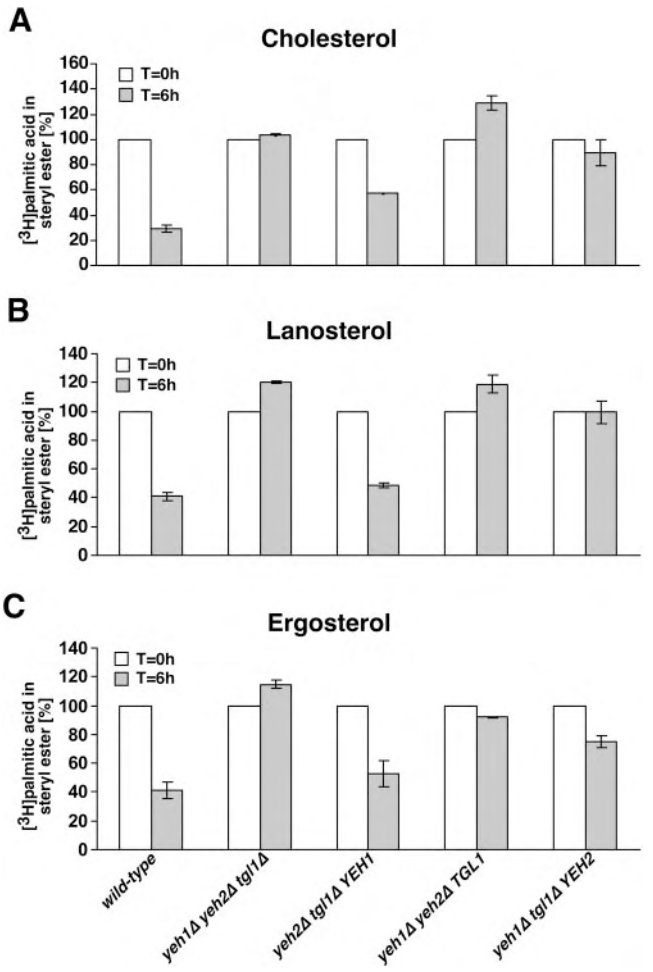

FIG. 3. Yeh1 is active against different steryl ester substrates. Heme-deficient wild-type (YRS1707), lipase triple mutant (YRS1922), and lipase double mutant (YRS1923, YRS1961, and YRS2045) cells were precultivated in media containing ergosterol, cholesterol, or lanosterol, and the steryl ester pool was labeled by incubating cells with [^{3}H]palmitic acid for 16 h. Cells were then diluted into fresh media containing the same sterol as that used for the precultivation, and samples were removed after 0 h and 6 h of growth. Lipids were extracted and analyzed by TLC, and levels of [^{3}H]palmitate in cholesteryl, lanosteryl, and ergosteryl esters were quantified by radioscanning. Values represent means and standard deviations from two independent experiments.

Heme deficiency results in increased steady-state levels of Yeh1. To begin to characterize the molecular mechanism(s) that is responsible for the high in vivo activity of Yeh1 and the apparent inactivity of Yeh2 and Tgl1 under heme deficiency, we first examined whether heme deficiency affects the subcellular distribution or steady-state levels of these three lipases. *HEM1* deficiency can be bypassed by supplementing the cells with the enzymatic product of the Hem1-catalyzed first step in heme biosynthesis, ALA (5). Thus, when supplemented with ALA, *hem1*Δ mutant cells have a normal aerobic metabolism and are capable of growing on nonfermentative carbon sources. To examine whether heme deficiency induces an altered subcellular localization or expression level of the lipases, heme-deficient cells expressing a functional, GFP-tagged version of the lipases were cultivated in media containing ALA or cholesterol plus Tween 80, and the subcellular localization of the lipases was examined by fluorescence microscopy. This analysis revealed that Yeh1 and Tgl1 localize to lipid particles under heme-proficient and heme-deficient conditions, indicating that these two lipases do not change their subcellular localization in response to heme status (Fig. 4A) (11). Yeh2, on the other hand, was previously localized to the plasma membrane in heme-proficient cells and displayed no alteration in the subcellular distribution upon heme depletion, again indicating that the subcellular localization of Yeh2 is not affected by the heme status of the cell (Fig. 4A) (11). The strong vacuolar staining observed here with the C-terminally GFP-tagged Yeh2 is likely due to vacuolar turnover of this fusion protein and is not observed with an N-terminal GFP fusion (11).

To examine whether heme depletion affects the steady-state levels of these lipases, strains expressing C-terminally GFP-tagged lipases from their native promoters were precultured in media supplemented with either ALA or cholesterol and diluted into fresh media containing the same supplements, and

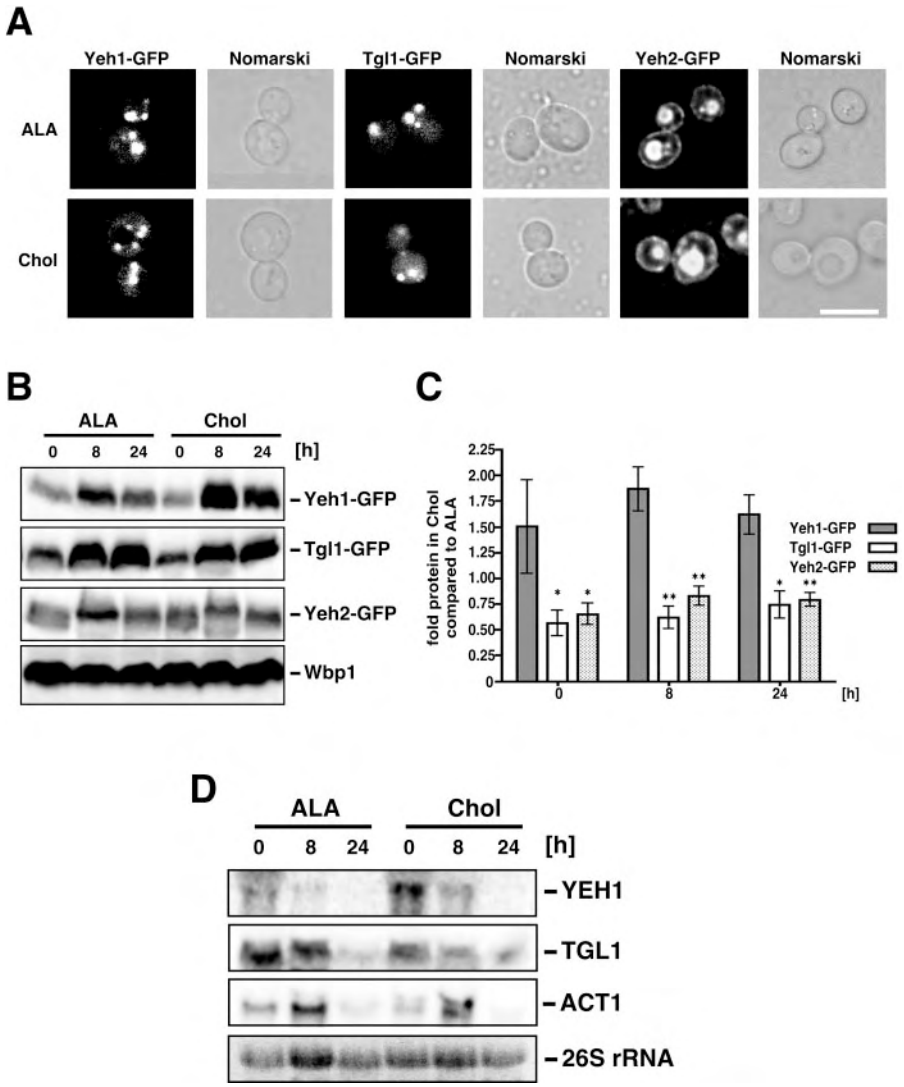

FIG. 4. Heme deficiency results in increased steady-state levels of Yeh1 at the expense of Yeh2 and Tgl1. (A) Heme-deficient cells expressing a GFP-tagged version of Yeh1 (YRS2046), Yeh2 (YRS2048), and Tgl1 (YRS2047) were cultivated in media containing either ALA or cholesterol (Chol) plus Tween 80 for 16 h, and the subcellular localization of the lipases was examined by fluorescence microscopy. Bar, 5 μm. (B) Heme-deficient cells expressing GFP-tagged lipases were precultivated in media containing either ALA or cholesterol plus Tween 80 for 16 h and diluted to an OD_{600} of 0.8, and samples were removed at the indicated time points. Steady-state levels of the GFP-tagged enzymes were analyzed by Western blotting, using Wbp1 as a loading control. (C) Signal intensities on Western blots were quantified by densitometry. Data represent means ± standard errors of the means ($n = 3$). Significance of the difference between the steady-state levels of Yeh1-GFP and Tgl1-GFP and between Yeh1-GFP and Yeh2-GFP, as based on a two-tailed unpaired t test, is indicated by asterisks (*, $P < 0.05$; **, $P < 0.01$). (D) Northern analysis of transcript levels. Heme-deficient wild-type cells (YRS1707) were cultivated in media containing either ALA or cholesterol plus Tween 80 for 16 h and diluted to an OD_{600} of 0.8, and samples were removed at the indicated time points. RNA was extracted, and transcript levels of *YEH1*, *TGL1*, and actin (*ACT1*) were determined by Northern blotting.

steady-state levels of the tagged lipases were examined by Western blotting at 0, 8, and 24 h after cell dilution. Protein loading was normalized to Wbp1, an ER-localized subunit of the oligosaccharyltransferase complex (Fig. 4B) (23). This analysis revealed that Yeh1 levels are approximately 1.5-fold higher in cholesterol-grown cells than in cells cultivated in ALA. Under these conditions, levels of Tgl1, on the other hand, are slightly decreased whereas those of Yeh2 remain constant. These data would thus indicate that the differential regulation of the in vivo activity of these three steryl ester hydrolases is mediated, at least in part, by an upregulation of the steady-state levels of Yeh1. These differences in steady-state levels of the enzymes are not due to differences in the growth phase of the cells analyzed, as they grew with comparable rates. The observed upregulation of Yhe1, however, cannot account solely for the strong dependence of steryl ester hydrolysis on Yeh1, as both Tgl1 and Yeh2 are present and only slightly downregulated. Upregulation of Yeh1 levels under heme-deficient conditions is due largely to increased steady-state levels of *YEH1* transcripts as revealed by Northern analysis, indicating that Yeh1 expression is subject to heme-dependent regulation (Fig. 4D).

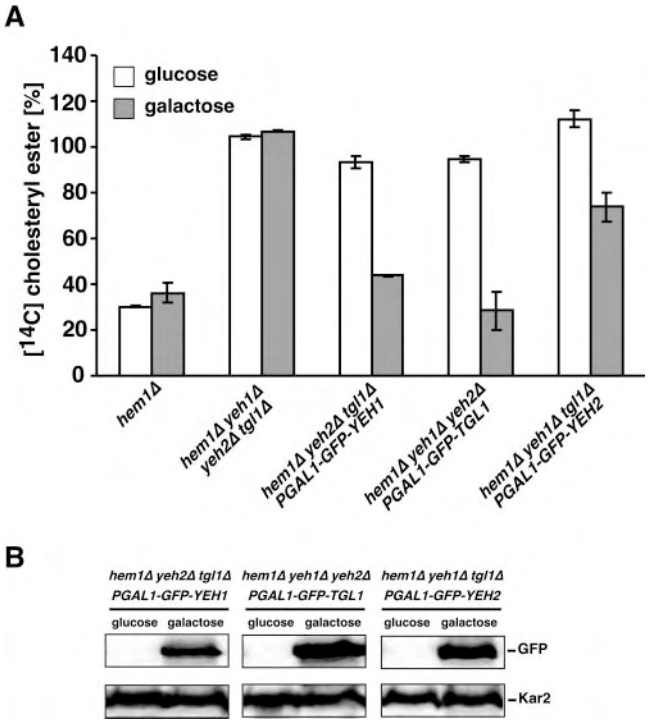

FIG. 5. The heme-dependent inactivation of Yeh2 and Tgl1 is overcome by overexpression of the enzymes. (A) Heme-deficient wild-type cells (YRS1707), *yeh1*Δ *yeh2*Δ *tgl1*Δ triple mutant cells (YRS1922), and cells expressing an N-terminally GFP-tagged version of Yeh1 (YRS2184), Yeh2 (YRS2183), and Tgl1 (YRS2182) from a regulatable *GAL1* promoter were labeled for 16 h with [^{14}C]cholesterol, and the kinetics of steryl ester mobilization was analyzed after dilution of cells into fresh media containing either glucose or galactose. Cells were cultivated for 6 h, lipids were extracted and analyzed by TLC, and the relative content of [^{14}C]cholesterol in the steryl ester pool was quantified by radioscanning of TLC plates. Values represent means and standard deviations from two independent experiments. (B) The level of repression or induction of the GFP-tagged lipases under the experimental conditions used for panel A was monitored by Western blot analysis, using Kar2 as a loading control.

Heme-dependent regulation of steryl ester hydrolysis is overcome by overexpression of Tgl1 and Yeh2. To test whether transcriptional regulation of the three lipases could account for the observed heme-dependent regulation, we examined whether overexpression of the lipases would overcome the apparent heme-dependent inactivation of Yeh2 and Tgl1. Therefore, expression of the three lipases was placed under the control of the inducible *GAL1* promoter. The in vivo activity of every one of the three lipases was then examined in the absence of the other two enzymes, i.e., in a lipase double mutant background. This analysis revealed that expression of each of the lipases is sufficient to induce steryl ester mobilization under heme-deficient conditions, as cells expressing the respective lipase display efficient mobilization of steryl esters when grown in galactose-containing media, i.e., under conditions where the promoter is active, but lack detectable steryl ester mobilization when cultivated in glucose-containing media, conditions that result in repression of the *GAL1* promoter (Fig. 5). These results thus indicate either that expression of Tgl1 and Yeh2 is rate limiting under heme-deficient conditions or that a possible negative regulation at the posttranslational level is overcome by the strong overexpression of these enzymes.

Heme-dependent induction of Yeh1 requires *ROX3*. The expression of hypoxic genes under aerobic conditions is repressed by Rox1, whereas Hap1 positively regulates expression of normoxic genes (12; for a review, see reference 29). We have previously observed that mutants that lack Rox3, a component of the mediator complex that controls the activity of RNA polymerase II, have elevated levels of steryl esters under anaerobic conditions (6, 18, 19). We thus examined whether Rox3 is required for the induction of Yeh1 under heme deficiency. Therefore, heme-deficient *rox3*Δ mutant cells were labeled with [^{14}C]cholesterol and mobilization of the radiolabeled steryl ester pool was examined over time. This analysis revealed that *rox3*Δ mutant cells were completely blocked in steryl ester mobilization (Fig. 6A). Analysis of the steady-state levels of Yeh1-GFP in the *rox3*Δ mutant background revealed that levels of this lipase are reduced under heme-deficient conditions, consistent with a requirement of Rox3 for a heme-dependent induction of Yeh1 (Fig. 6B).

Taken together, these data indicate that the heme-dependent activity of Yeh1 at the expense of Tgl1 and Yeh2 is mediated at least in part by upregulation of the steady-state levels of Yeh1 and a concomitant decrease of Tgl1 and Yeh2 in exponentially growing cells and that the mediator component Rox3 is required for this upregulation of Yeh1.

DISCUSSION

Levels of steryl esters are coordinated with the growth phase (1, 22). It is thus necessary that their synthesis or mobilization or both are tightly regulated. As an important step towards understanding sterol homeostasis at a cellular level, the aim of this study was to identify the in vivo role of the three partially redundant steryl ester hydrolases Yeh1, Yeh2, and Tgl1 under conditions that mimic anaerobiosis, i.e., heme deficiency. Our analysis indicates that under these conditions, Yeh1 is the major steryl ester hydrolase in vivo and that Tgl1 and Yeh2 do not significantly contribute to steryl ester mobilization in heme-deficient cells. Examination of the substrate specificities of the three lipases in vivo indicates that this differential regulation is not due to a preference of Yeh1 to hydrolyze steryl esters containing nonergosterol esters, which are known to accumulate under anaerobic or heme-deficient conditions and whose potential toxic effect is remedied by selective esterification by Are1, as Yeh1 is equally active against cholesteryl and lanosteryl esters as it is against ergosteryl esters (5, 10, 24).

Heme deficiency does not affect the membrane association, subcellular localization, or apparent molecular weight of Yeh1, Yeh2, and Tgl1 when analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis of GFP-tagged fusions (data not shown), suggesting that the heme-dependent activity of Yeh1 and apparent inactivity of Tgl1 and Yeh2 are not due to a relocalization of the enzymes. Heme deficiency, however, results in an increase in steady-state levels of Yeh1 and a concomitant downregulation of Tgl1 and Yeh2 in exponentially growing cells. Differential regulation of enzyme levels might thus at least in part explain the differential activation of Yeh1 under heme deficiency. The fact that Tgl1 and Yeh2 steady-state levels are not more strongly reduced under heme

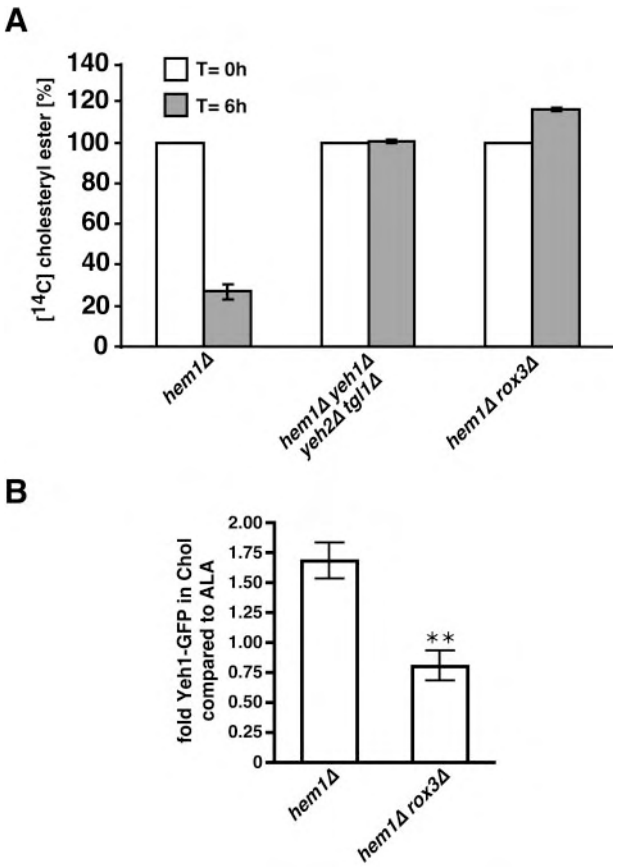

FIG. 6. *ROX3* is required for efficient mobilization of steryl esters under heme deficiency. (A) Heme-deficient wild-type (YRS1707), lipase triple mutant (YRS1922), and *rox3*Δ mutant (YRS1766) cells were labeled for 16 h with [^{14}C]cholesterol, and the kinetics of steryl ester mobilization in vivo was analyzed by determining steryl ester levels at 0 and 6 h after the dilution of cells into fresh media. Lipids were extracted and analyzed by TLC, and the relative content of [^{14}C]cholesterol in the steryl ester pool was quantified by radioscanning of TLC plates. Values represent means and standard deviations from two independent experiments. (B) *ROX3* is required for induction of Yeh1-GFP in lipid-supplemented media. Heme-deficient wild-type cells (YRS2046) and *rox3*Δ mutant cells expressing Yeh1-GFP (YRS2740) were cultivated for 24 h in media containing either ALA or cholesterol plus Tween 80, and levels of Yeh1-GFP were determined by quantification of Western blots, using Wbp1 as a loading control. Signal intensities were quantified by densitometry. Data represent means ± standard errors of the means ($n = 4$). Significance of the difference between the steady-state levels of Yeh1-GFP in wild-type and *rox3*Δ mutant cells, as based on a two-tailed unpaired *t* test, is indicated by asterisks ($P = 0.0042$).

deficiency, however, would indicate that these enzymes are inactivated at the posttranslational level. Such an inactivation mechanism may be overcome by strong overexpression of the two enzymes, explaining why the overexpression of Tgl1 and Yeh2 from the *GAL1* promoter restores their activity in vivo. Cycloheximide chase experiments revealed that upregulation of Yeh1 under heme-deficient conditions does not affect the turnover of the enzyme, indicating that upregulation is due largely to increased expression of *YEH1* (data not shown). Consistent with such a transcriptional regulation of Yeh1, we find that Rox3, a component of the mediator complex that is required for both activation and repression of RNA polymerase II activity, is required for steryl ester hydrolysis under heme deficiency and that cells lacking *ROX3* have reduced steady-state levels of Yeh1.

How exactly the lipases gain access to their substrates and how this step is regulated are not yet well understood. It is, however, interesting to note that under conditions where Tgl1 is inactive, the enzyme is still present and localized to lipid particles, suggesting that it lacks the signal required for its activity. Our observation that this activating signal can be provided by overexpression of the enzyme may indicate that expression of the lipases is rate limiting for their in vivo activity. Thus, lipase activity in yeast may be controlled primarily at the transcriptional level. This would be in contrast to the situation of mammalian cells in which an interplay between components that localize to the lipid droplets, such as perilipin and the lipase, in this case the hormone-sensitive lipase, is important in coordinating substrate access. This interplay is regulated by protein kinase A to increase the rate of lipolysis 30- to 100-fold (21).

Even though yeast lipid particles lack any obvious perilipin orthologue, it is interesting to note that both Yeh1 and Tgl1, but not Yeh2, contain potential cyclic AMP-dependent protein kinase A phosphorylation sites. Additional studies will now be required to determine how the activity and substrate access of the yeast lipases are regulated.

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Additional Experiments

1. Introduction

1.1. *Saccharomyces cerevisiae* and sporulation

Sporulation in budding yeast constitutes a specialized developmental pathway in which diploid cells undergo meiosis to produce four haploid germ cells, so called spores (for recent review see 1, 2). These spores are then able to fuse with cells of the opposite mating type, a process related to the fusion of egg and sperm. There exist at least four sets of genes, early, middle, mid-late, and late, which are sequentially transcribed during sporulation. Early genes are important for the meiotic prophase in which pairing of homologue chromosomes and recombination occurs. So called middle genes in sporulation participate in meiotic nuclear division as well as in spore formation, whereas mid-late genes are required for the formation of the spore wall. Finally, genes of the late class are necessary for spore maturation. However, many regulating factors as well as other gene products required for sporulation still remain unknown and are subject of extensive research. Recently, neutral lipid biogenesis as well as mobilization was implicated to play a role in this developmental pathway. For example, deletion of the STE synthesizing enzymes Are1 and Are2 affects the sporulation efficiency in homozygous diploids (5). Not surprisingly, homozygous diploids deleted for *TGL1*, which encodes a STE hydrolase required for STE mobilization, are also unable to sporulate (6). Furthermore, not only STE, but also TAG seems to be important for sporulation as e.g. mutants unable to mobilize TAG show severe sporulation defects (3). In line with these findings, the yeast TAG hydrolase Tgl3p as well as its homologues were found to be highly expressed during sporulation (4). Another interesting finding came from the analysis of the transcriptional program of sporulation in yeast, where a STE hydrolase, namely *YEH1*, showed increased expression in the middle phase of sporulation (7). To verify a role of this particular lipase in the sporulation process, homozygous and heterozygous *YEH1* deleted diploids where phenotypically analysed in more detail.

1.2. *Saccharomyces cerevisiae* and anaerobiosis

The budding yeast, *Saccharomyces cerevisiae*, is a facultative anaerobic organism that becomes auxotroph for sterols and unsaturated fatty acids in the absence of oxygen. In presence of oxygen the fungal ergosterol is endogenously synthesized and cells do not take up exogenous sterol. This phenomenon is called "aerobic sterol exclusion." However, in the absence of oxygen, under anaerobic conditions yeast becomes auxotrophic for sterols and unsaturated fatty acids, because their synthesis requires molecular oxygen (8). Thus, under anaerobic conditions exogenous sterols have to be taken up from the environment. Another

way to mimic anaerobiosis in yeast is to delete the cells for *HEM1*, which encodes for an enzyme that catalyzes the first step of heme biosynthesis. Lack of heme then leads to uptake of exogenous sterols even in presence of oxygen (9). We showed that one of the three STE hydrolases of yeast, Yeh1p exhibits activity under heme-deficient conditions (10). In the following experiments we monitored the growth (i) of STE hydrolase single-, double-, and triple-deletion strains under anaerobic conditions on agar plates supplemented with different sterols, and (ii) of heme-deficient STE hydrolase mutants in liquid media supplemented with cholesterol. The overall purpose was to examine if these lipase mutants, especially mutants deleted for *YEH1*, are affected when grown in presence of excess sterol, because it has been shown recently that accumulation of free cholesterol in the ER of macrophages induces cellular stress and finally leads to apoptosis (11).

2. Materials and Methods

2.1. Strains, media, and growth conditions

Yeast strains used in this study are listed in Table 1. Single deletion mutants were obtained from EUROSCARF (www.rz.unifrankfurt.de/FB/fb16/mikro/euroscarf/index.html), and double and triple-mutant strains were generated by gene disruption with PCR deletion cassettes or by crossing of single-mutant strains (12). Homozygous and heterozygous diploids were created by crossing haploid single-mutants and GFP-tagged strains with each other, or with wild-type BY4742, or BY4741, or were obtained from EUROSCARF (14).

2.2. Growth test under anaerobic conditions

For plate assays on sterol containing media, fresh overnight cultures were inoculated into YPD (1% Bacto Yeast Extract, 2% Bacto Peptone [U.S. Biological, Swampscott, Mass.], 2% glucose) to OD_{600} of 0.2 and grown to OD_{600} of ~1 at 24°C or 30°C. Next, equivalents of 2 OD_{600} units were harvested, resuspended in 200 µl fresh SC+All, and serial 10-fold dilutions were spotted on agar plates without sterol, or supplemented with either 20 µg/ml ergosterol or cholesterol and 5 mg/ml Tween 80 as a source for fatty acids. Agar plates were incubated under anaerobic conditions using an anaerobic jar containing an AnaeroGen sachet (Oxoid, Basingstoke, Hampshire, England) for up to 5 days at 24°C before examination.

2.3. Sporulation assay

Diploid cells were grown on YPD medium overnight and transferred to SPM (0.3% potassium acetate, 0.02% raffinose) to induce sporulation. Cells were incubated at 24°C and sporulation

was then monitored by differential interference contrast (DIC) microscopy. For analysis of GFP fusion proteins, live cells were examined with a Zeiss Axioskop 2 fluorescence microscope (Carl Zeiss, Oberkochen, Germany).

Table 1: *Saccharomyces cerevisiae* strains used in this study

Strain	Relevant genotype	Source or reference
YRS1533	BY4742; *MATα his3Δ1 leu2Δ0 ura30 lys2Δ0*	EUROSCARF; 14
YRS1972	*MATα his3Δ1 leu2Δ0 ura30 lys2Δ0 yeh1*::*kanMX4*	EUROSCARF; 14
YRS1971	*MATα his3Δ1 leu2Δ0 ura30 lys2Δ0 yeh2*::*kanMX4*	EUROSCARF; 14
YRS1973	*MATα his3Δ1 leu2Δ0 ura30 lys2Δ0 tg11*::*kanMX4*	EUROSCARF; 14
YRS1948	*MATα his3Δ1 leu2Δ0 ura30 lys2Δ0 yeh1*::*HIS3MX6 yeh2*::*kanMX4*	This study
YRS1837	*MATα his3Δ1 leu2Δ0 ura30 lys2Δ0 yeh1*::*HIS3MX6 tg11*::*kanMX4*	This study
YRS1838	*MAT? his3Δ1 leu2Δ0 ura3Δ0 met15Δ0 LYS2 yeh2::kanMX4 tg11::kanMX4*	This study
YRS1840	*MAT? his3Δ1 leu2Δ0 ura3Δ0 met15Δ0 LYS2 yeh1::HIS3MX6 yeh2::kanMX4 tg11::kanMX4*	This study
yeh1Δ / yeh1Δ	*BY4743; Mat a/α; his3Δ1/his3Δ1; leu2Δ0/leu2Δ0; lys2Δ0/LYS2; MET15/met15Δ0; ura3Δ0/ura3Δ0; yeh1::kanMX4/yeh1::kanMX4*	EUROSCARF; 14
yeh1Δ / YEH1-GFP	*Mat a/α; his3Δ1/his3Δ1; leu2Δ0/leu2Δ0; lys2Δ0/LYS2; MET15/met15Δ0; ura3Δ0/ura3Δ0; yeh1::kanMX4/YEH1-GFP-HIS3MX6*	This study
YEH1 / YEH1	*Mat a/α; his3Δ1/his3Δ1; leu2Δ0/leu2Δ0; lys2Δ0/LYS2; MET15/met15Δ0; ura3Δ0/ura3Δ0; YEH1/YEH1-GFP-HIS3MX6*	This study
YRS1710	BY4742; *MATα his3Δ1 leu2Δ0 ura30 lys2Δ0 hem1::LEU2*	This study
YRS1922	*MAT? his3Δ1 leu2Δ0 ura3Δ0 met15Δ0 LYS2 yeh1::HIS3MX6 yeh2::kanMX4 tg11::kanMX4 hem1::LEU2*	This study
YRS1961	*MAT? his3Δ1 leu2Δ0 ura3Δ0 met15Δ0 LYS2 yeh2::kanMX4 tg11::kanMX4 hem1::LEU2*	This study
YRS2045	*MATα his3Δ1 leu2Δ0 ura30 lys2Δ0 yeh1*::*HIS3MX6 yeh2*::*kanMX4 hem1::LEU2*	This study
YRS1923	*MATα his3Δ1 leu2Δ0 ura30 lys2Δ0 yeh1*::*HIS3MX6 tg11*::*kanMX4 hem1::LEU2*	This study

2.4. Growth on cholesterol containing media

Heme-deficient cells were grown in YPD supplemented with 20 µg/ml delta-aminolevulinic acid (ALA) overnight at 24°C. Next, the culture was harvested, washed twice with YPD to remove residual ALA, and diluted into fresh YPD with ALA, or supplemented with 20 µg/ml cholesterol, 0.5% Tween 80 to OD_{600} of 0.3. Cultures were incubated at 30°C and growth was determined at indicated time points. In addition, at each time point the culture was diluted again to OD_{600} of 0.3 into fresh media and incubated again for 8 hours or 16 hours to document the growth capacity.

3. Results

3.1. The STE hydrolase Yeh1 is not required for efficient sporulation

The STE hydrolase Tgl1p is required for sporulation in yeast and *YEH1* shows increased expression during sporulation (6, 7). To investigate if the STE hydrolase Yeh1p is also necessary for sporulation, a homozygous *yeh1Δ/yeh1Δ* diploid strain was tested for sporulation efficiency. As can be seen in Fig.1A, a *yeh1Δ/yeh1Δ* diploid is able to form spores with efficiency comparable to wild-type.

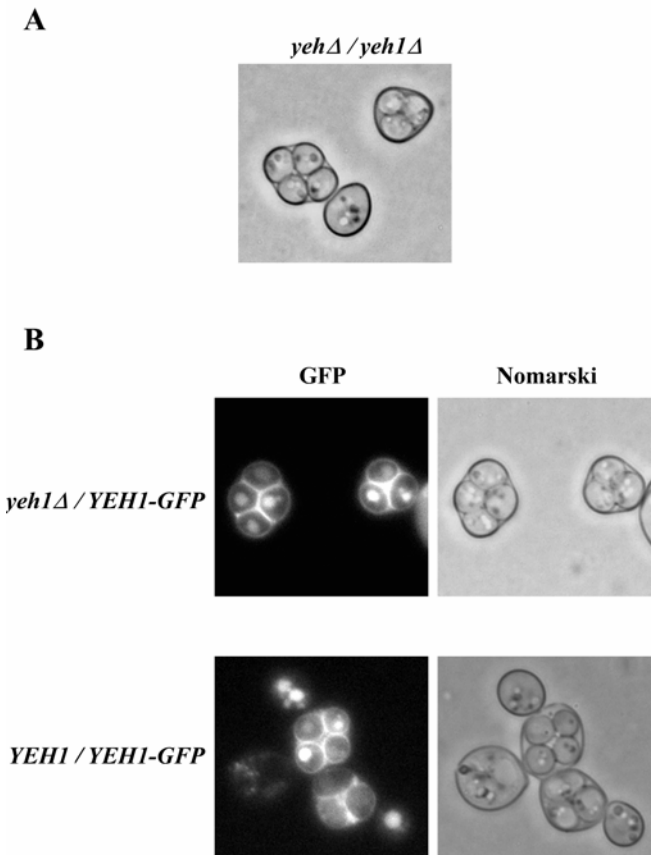

Figure 1: A *yeh1Δ/yeh1Δ* diploid strain is not affected in sporulation. Diploid strains were grown on YPD overnight and transferred to SPM to induce sporulation. Efficiency of sporulation was monitored using DIC microscopy. (A) A diploid STE hydrolase *yeh1Δ/yeh1Δ* deletion mutant shows normal sporulation pattern. (B) Localization studies of Yeh1p-GFP in sporulated diploid strains (*yeh1Δ/YEH1-GFP*; *YEH1/YEH1-GFP*;) reveal intense staining of membranes in all four spores of the ascus.

Not surprisingly, a heterozygous *YEH1/yeh1Δ* strain also showed no defects in sporulation (Fig.1B, upper panel). In addition, the localization of Yeh1p-GFP was assessed in ascospores using one copy of *YEH1* chromosomally tagged with GFP in diploids. The second genomic copy of *YEH1* was either deleted or left wild-type. Microscopic examination showed that Yeh1p-GFP localized to the membrane of the four haploid spores in the ascus, regardless of the presence of a second functional copy of *YEH1* (Fig.1B). The round shaped fluorescent signal inside the haploid spores does not selectively appear in the green channel and thus is most likely not GFP but due to autofluorescence. Taken together, the results indicate that the STE hydrolase Yeh1p is not necessary for the sporulation process in yeast.

3.2. Growth of STE hydrolase mutants is not affected on sterol media under anaerobic conditions

To examine if strains which are unable to mobilize STE are sensitive to excess sterol, we have grown single-, double-, and triple-STE hydrolase mutant strains on agar plates supplemented with ergosterol or cholesterol. Under anaerobic conditions, yeast becomes auxotrophic for sterols and thus takes up exogenous sterol from the environment. The basic idea was to test if it was possible to "overload" the cells with exogenous sterols when STE hydrolysis is blocked. Such an overload is expected to result in growth arrest. As shown in Fig.2, single-, double-, and triple-STE hydrolase deleted strains show growth patterns comparable to wild-type on media containing ergosterol or cholesterol.

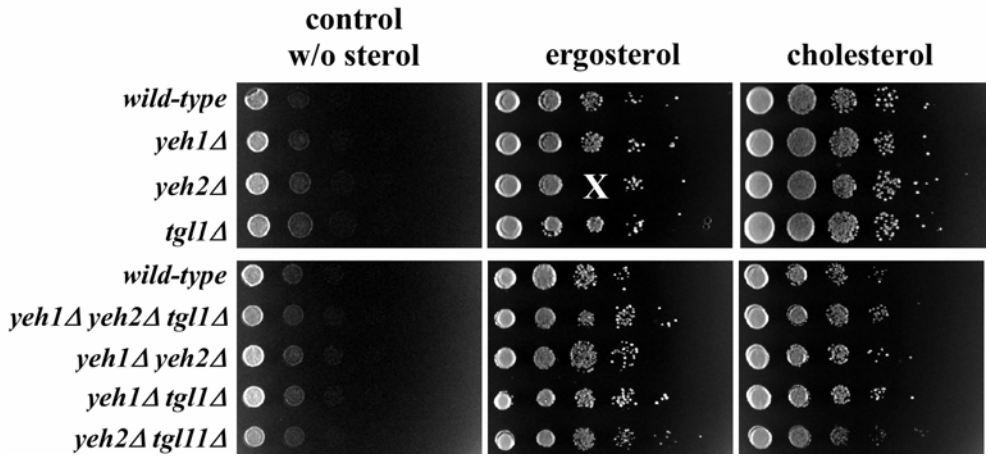

Figure 2: Growth of STE hydrolase mutants on sterol supplemented solid media under anaerobic conditions. Wild-type (BY4742, YRS1533), lipase single-mutant (*yeh1Δ*, YRS1972; *yeh2Δ*, YRS1971; *tgl1Δ*, YRS1973), double-mutant (*yeh1Δ yeh2Δ*, YRS1948; *yeh2Δ tgl1Δ*, YRS1838; *yeh1Δ tgl1Δ*, YRS1837), and triple-mutant (*yeh1Δ yeh2Δ tgl1Δ*, YRS1840) cells were spotted onto media containing no sterol, ergosterol or cholesterol (20 µg/ml). Anaerobic conditions were maintained using an anaerobic jar and images were taken after 5 days of incubation at 24°C.

It has to be taken into account, however, that the cells stop to take up exogenous sterol over time under this experimental conditions, which allows no definite answer to the question if excess sterol from the environment is toxic for yeast cells. The observation that a *yeh1Δ* single- as well as a *yeh1Δ yeh2Δ tgl1Δ* triple-lipase mutant is viable under anaerobic conditions indicates that STE mobilization is not essential under these conditions (Fig.2).

3.3. STE hydrolase deletion mutants have reduced doubling time on media supplemented with cholesterol

Here we wanted to test the growth behaviour of heme-deficient STE hydrolase mutants under conditions where they are continuously grown in cholesterol containing media. As mentioned above, lack of heme enables cells to take up exogenous sterols even in the presence of oxygen. In addition, heme deficiency caused by deletion of *HEM1* can be bypassed by supplementing the cells with ALA, the product of Hem1p (13). In other words, cells supplemented with ALA display aerobic growth, whereas cells supplemented with cholesterol behave as if they are in anaerobic conditions. Furthermore, the continuous cultivation of *hem1Δ* mutants on cholesterol should deplete cells over time for ergosterol which gets replaced by the supplemented animal sterol. The growth behaviour of these mutant strains in either ALA or cholesterol containing media is shown in Fig.3. Interestingly, STE hydrolase mutant cells supplemented with ALA, which mimics aerobic conditions, also show retarded growth after the second passage into fresh media as compared to wild-type (upper graph). However, STE hydrolase mutant strains show strongly reduced doubling times when grown in media supplemented with cholesterol (lower graph). The *hem1Δ* wild-type seems not to be affected when grown on cholesterol. On the other hand, STE hydrolase double- and triple-deletion mutants grow much slower. When calculated from the OD_{600} values that they reached after 8 or 16 hours, the doubling time of the STE hydrolase mutant strains is approximately four-times longer than that of wild-type. These results can be interpreted in two ways. First, cholesterol accumulates in excess in cells affected in STE mobilization, thus slowing down growth, and second, which is more likely, STE mobilization is needed to support rapid initiation of growth when cells are diluted into fresh media. To discriminate between these two possibilities additional experiments are required, such as to examine the growth of a strain deficient for STE synthesis, which accumulates unesterified sterols.

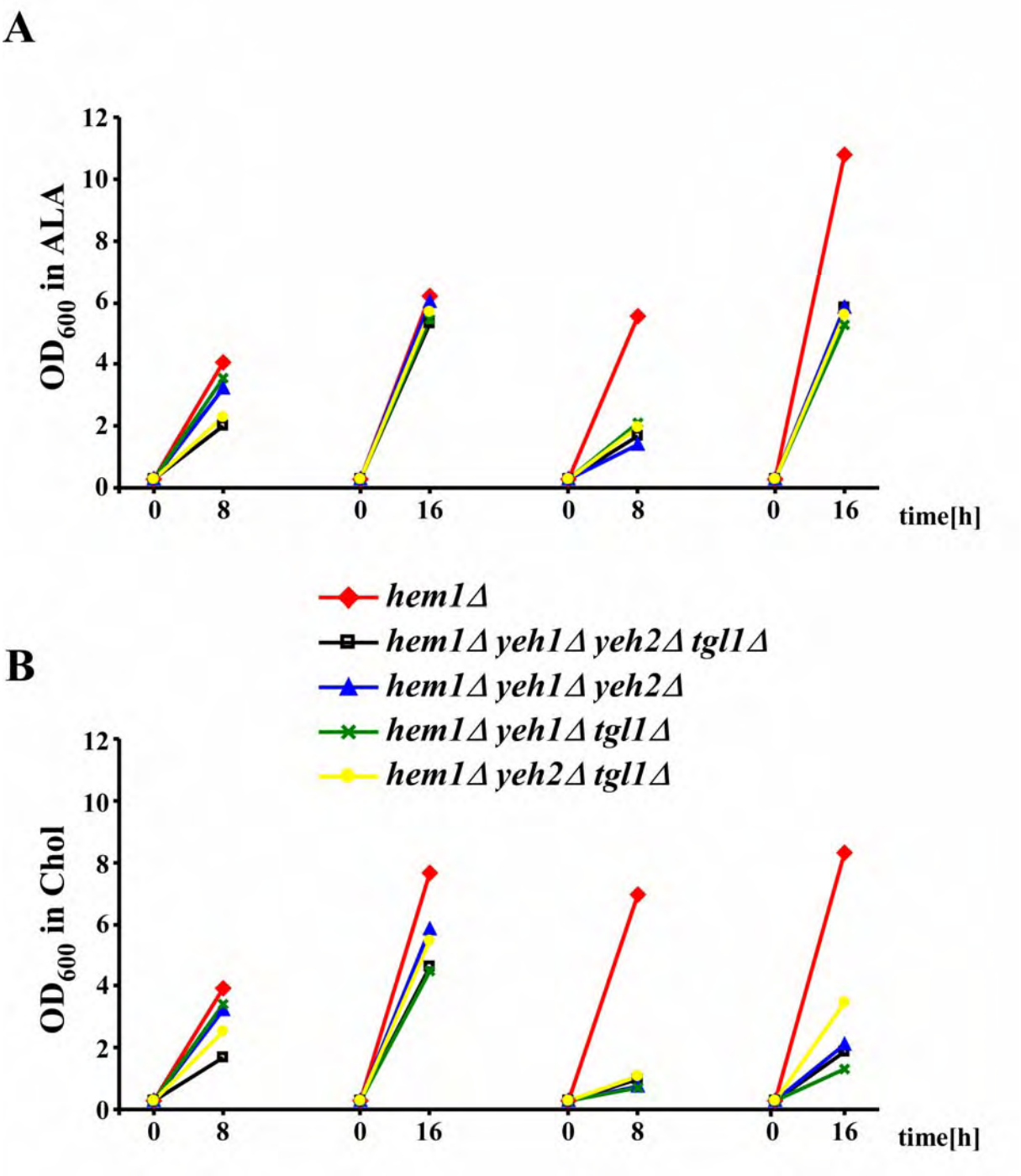

Figure 3: STE hydrolase deletion mutants show reduced doubling time during continuous growth in media containing cholesterol. Heme-deficient wild-type (BY4742 *hem1Δ*, YRS1710), lipase double-mutant (*hem1Δ yeh1Δ yeh2Δ*, YRS2045; *hem1Δ yeh2Δ tgl1Δ*, YRS1961; *hem1Δ yeh1Δ tgl1Δ*, YRS1923), and triple-mutant (*hem1Δ yeh1Δ yeh2Δ tgl1Δ*, YRS1922) cells were grown in YPD supplemented with either 20 µg/ml ALA or 20 µg/ml cholesterol at 24°C. After 8 hours, density of the culture was determined and cultures were diluted to OD_{600} of 0.3 into fresh media and grown for another 16 hours. Cells were again diluted and density of the culture was determined after 8, and 16 hours.

4. Discussion

The aim of these studies was to examine the cell biological role of STE hydrolases in yeast. Recently it has been shown that neutral lipid metabolism is important to promote sporulation. Mutant *are1Δ are2Δ* strains, deficient for synthesis of STE, show reduced sporulation efficiency (5). Moreover, a *tgl1Δ/tgl1Δ* STE hydrolase diploid mutant is also unable to sporulate, which strengthens the view that STE are important for this developmental process. Interestingly, a second STE hydrolase, encoded by *YEH1*, was shown to be highly expressed during sporulation. Microarray studies analyzing changes of transcript levels of various stages of sporulation revealed that transcript levels for *YEH1* are elevated 14-fold during the middle phase of the sporulation process, indicating that this STE hydrolase is probably important for this process (7). Thus, we examined whether Yeh1p deficiency affects sporulation. Our result indicates that a homozygous *yeh1Δ/yeh1Δ* diploid is not defective for sporulation, which shows that Yeh1p seems to be dispensable for this process. Thus, the cause for the increased transcription of this lipase in the middle phase of the sporulation program cannot be explained by a requirement of Yeh1p for sporulation. We also started to investigate the relevance of STE hydrolases under anaerobic conditions, because recent work from our lab indicates that only the STE hydrolase Yeh1p is active under these conditions (10). As mentioned above, when maintained under anaerobic conditions yeast has to take up exogenous sterols from the environment. As we wanted to examine growth defects of STE mobilization deficient cells, when challenged with excess of exogenous sterol, we had to cultivate yeast under oxygen depleted conditions. For this purpose we used an anaerobic jar and tested the growth of single-, double-, and triple-lipase mutants on media supplemented either with ergosterol or cholesterol. STE hydrolase mutants did not fail to grow under anaerobic conditions. This indicates that Yeh1p, although it is the only active STE hydrolase under these conditions, is not essential for growth under oxygen-depletion. Furthermore, even a *yeh1Δ yeh2Δ tgl1Δ* triple-mutant is viable under these conditions, and not affected by excess of exogenous sterols. The working hypothesis was that uptake of high amounts of sterols may lead to cellular stress and cell death as observed in macrophages (11). However, it is more likely that yeast does not take up excess amounts of sterols, even under anaerobic conditions. A block in heme biosynthesis, achieved by deletion of *HEM1*, also enables yeast to take up exogenous sterols even in the presence of oxygen. We thus tested growth of heme-deficient STE hydrolase mutants on cholesterol supplemented media. This time the basic idea was to continuously grow the cells in cholesterol for several generations and heme-deficient strains continuously grown on cholesterol may start to replace their endogenous ergosterol with the

animal sterol. Our results revealed that a *hem1Δ* wild-type is not affected when grown on cholesterol for several generations. On the other hand, heme-deficient STE hydrolase mutants show retarded growth on cholesterol, with a doubling time four-times longer than that observed for *hem1Δ* alone. When these mutants were grown in media supplemented with ALA, slower growth of the lipase mutants was also observed, but it was not as pronounced as when grown in cholesterol. The results of these experiments suggest that STE mobilization is important for rapid initiation of growth when cells are diluted into fresh media, regardless of whether the cells grow under aerobic (with ALA) or anaerobic (with cholesterol) conditions. Moreover, growth retardation is more drastic in lipase mutant cells grown on cholesterol, one could thus speculate that cholesterol alone is maybe not suitable to fulfil all functional requirements of ergosterol, when this gets finally replaced after several generations. The STE hydrolase mutants are not able to "use" the STE pool and thus are dependent on cholesterol which is imported from the environment. To test this hypothesis the experiment has to be repeated using ergosterol instead of cholesterol. Moreover, it is likely that a wild-type strain, able to utilize stored STE, has an energy advantage at low OD_{600} for a rapid start of logarithmic growth.

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CHAPTER IV

Phosphorylation of Yeh2p and identification of the respective protein kinases

1. Introduction

Neutral lipids, such as TAG and STE, serve as a storage form for fatty acids and sterols in eukaryotes and specialized lipases are required for mobilization of these lipids. The enzymatic hydrolysis of these stored neutral lipids has to be tightly regulated. The posttranslational modification of lipases by phosphorylation plays a central role in regulation of their enzymatic activity. For example in adipocytes, the mammalian neutral lipid lipase HSL gets highly phosphorylated upon lipolytic stimulation which results in a ~100-fold increase in its activity (1, 2). Moreover, phosphorylation of HSL promotes its translocation from the cytoplasma to the lipid droplet (11). Recently, we identified three STE hydrolases in yeast (3). In order to gain insight in the regulation of theses three STE hydrolases Yeh1p, Yeh2p, and Tgl1p we examined if there are any detectable posttranslational modifications such as phosphorylation on these proteins. The three lipases indeed possess numerous potential phosphorylation sites (see Table 2).

Table 2: Potential phosphorylation sites in Yeh1p, Yeh2p and Tgl1p

Steryl ester hydrolase	**Predicted phosphorylation sites ***		
Yeh1	Ser: 14	Thr: 9	Tyr: 6
Yeh2	Ser: 13	Thr: 5	Tyr: 4
Tgl1	Ser: 21	Thr: 6	Tyr: 5

* http://www.cbs.dtu.dk/services/NetPhos/

Dephosphorylation of the lipases with alkaline phosphatase *in vitro* revealed that Yeh2p is phosphorylated as indicated by a mobility shift of the protein in SDS-PAGE gel electrophoresis and western blot analysis. For the other two lipases Yeh1p and Tgl1p, however, no difference in mobility upon dephosphorylation with alkaline phosphatase could be detected. Phosphorylation of proteins is carried out by a family of protein kinases which add the phosphate group to Ser, Thr or Tyr in their target proteins. There are 116 protein kinases in yeast (http://www.mips.gsf.de). Of these, 13 are essential for viability (see Table 3), whereas the remaining 103 are none-essential (see Table 4), even though mutants lacking those protein kinases often have severe defects and multiple phenotypes e.g. mating deficiency and slow growth (http://www.yeastgenome.org). To identify the protein kinase(s) which phosphorylate Yeh2p we tempted to set up a screen in which we tagged Yeh2p with GFP in a none-essential protein kinase deletion strain collection and compared the electrophoretic mobility of Yeh2p-GFP in these mutant strains to wild-type by western blot analysis.

2. Materials and Methods

2.1. Strains, media, and growth conditions

None-essential protein kinase deletion strains were obtained from EUROSCARF (www.rz.uni-frankfurt.de/FB/fb16/mikro/euroscarf/index.html) and grown either on YPD (1% Bacto Yeast Extract, 2% Bacto Peptone [U.S. Biological, Swampscott, Mass.], 2% glucose), YPD containing 200 µg/ml G418 (Gibco BRL, Life Technologies) or minimal media at 24°C or 30°C. For tagging of Yeh2p with GFP the candidate protein kinase mutant strains were transformed with a Yeh2-GFP-HIS3MX6 PCR fusion cassette using standard procedures (3, 4). Yeast strains used in this study are listed in Table 1.

Table 1: *Saccharomyces cerevisiae* strains used in this study

Strain	Relevant genotype	Source or reference
YRS1974	*MATα his3Δ1 leu2Δ0 ura3Δ0 lys2Δ0 YEH1-GFP-HIS3MX6*	This study
YRS2086	*MATα his3Δ1 leu2Δ0 ura30 lys2Δ0 YEH2-GFP-HIS3MX6*	This study
YRS1858	*MATα his3Δ1 leu2Δ0 ura3Δ0 lys2Δ0 TGL1-GFP-HIS3MX6*	This study
YRS2499	*MATα his3Δ1 leu2Δ0 ura30 lys2Δ0 kcc4::kanMX4 YEH2-GFP-HIS3MX6*	This study
YRS2497	*MATα his3Δ1 leu2Δ0 ura30 lys2Δ0 vhs1::kanMX4 YEH2-GFP-HIS3MX6*	This study
YRS2500	*MATα his3Δ1 leu2Δ0 ura30 lys2Δ0 swe1::kanMX4 YEH2-GFP-HIS3MX6*	This study
YRS2498	*MATα his3Δ1 leu2Δ0 ura30 lys2Δ0 ynr047::kanMX4 YEH2-GFP-HIS3MX6*	This study

2.2. Colony PCR and western blot analysis

Colony PCR of selected transformants was performed as described earlier (3) using forward primer regYLRmid (5`- GTGAGGCTAATGGCAAAAGG -3´) and revGFPcontr (5`- TTCGGGCATGGCACTCTTGA - 3´) as a reverse primer. Positive clones were grown overnight in 2 ml YPD at 24°C and 2 OD_{600} were collected the next day. Proteins were extracted essentially as described (5) and proteins equivalent to 0.5 OD_{600} each were subjected to 8% SDS-PAGE. Separation of total protein on the SDS - gel was carried out at 40V – 50V constant for at least 4 h to achieve sufficient separation of proteins with minor different

molecular weight. Western blot was performed by standard procedures using antibodies against GFP (1:5,000; TorreyPines Inc.).

2.3. Alkaline phosphatase treatment of cell extracts

Cells were grown in 10 ml YPD over night at 24°C or 30°C and 2 OD_{600} were harvested the next day. Cell pellets were resuspended in 100 µl 0.2 M NaOH, 0.5% 2-mercaptoethanol and incubate on ice for 15 min. Next 1 ml ice cold acetone was added and proteins were precipitated for 30 min at -20°C. Proteins were pelleted (16,000 x g, 5 min, 4°C) and resuspended in 150 µl buffer A (50 mM Tris-HCl pH 8, 1 mM EDTA, 1% SDS). Next, the lysate was diluted with 1.9 ml buffer B (50 mM Tris-HCl pH 8, 1 mM $MgCl_2$, 0.5% TX-100) and centrifuged at 16,000 x g for 5 min at 4°C to remove insoluble proteins. The supernatant was divided into 2 fractions (1 ml each) and 2.5 U alkaline phosphatase (FLUKA; #79385) was added to one fraction. Next, both fractions were incubated at 37°C for 1 h and then precipitated by addition of TCA to a final concentration of 8%. TCA precipitated proteins were washed once with ice cold acetone, air dried, dissolved in 20 µl 1 x SDS loading dye, 2-mercaptoethanol, and analyzed by 8% SDS-PAGE and western blot using anti-GFP antibodies.

3. Results

3.1. Growth phase dependent phosphorylation of Yeh2p

Western blot analysis of protein extracts of strains expressing the three STE hydrolases tagged with GFP revealed that Yeh2p-GFP appeared not as a single band but showed multiple bands of different sizes within a range of about 10 kDa (Fig.1). The other two lipases, Yeh1p and Tgl1p, showed single bands by western blot analysis. The appearance of multiple Yeh2p-GFP bands as seen by SDS-PAGE and western blot analysis suggests that this was due to posttranslational modifications of Yeh2p. Since, we were not able to detect any glycosylation of Yeh2p (3) we tested the possibility that different phosphorylated forms of Yeh2p give rise to these multiple bands. Dephosphorylation of membrane fractions isolated from a wild-type strain bearing Yeh2-GFP by alkaline phosphatase treatment revealed that band-shift of Yeh2p-GFP in the phosphatase treated fractions occurs, which is likely to reflect a partially dephophorylated form of Yeh2p-GFP (Fig. 1). This suggests that the higher molecular weight forms of Yeh2p are phosphorylated, whereas the lower band(s) constitute partially dephosphorylated or none-phosphorylated forms. There was no evidence that Yeh1p and

Tgl1p were also phosphorylated since we did not obtain any changes in electrophoretic mobility of these proteins after alkaline phosphatase treatment (Fig. 1).

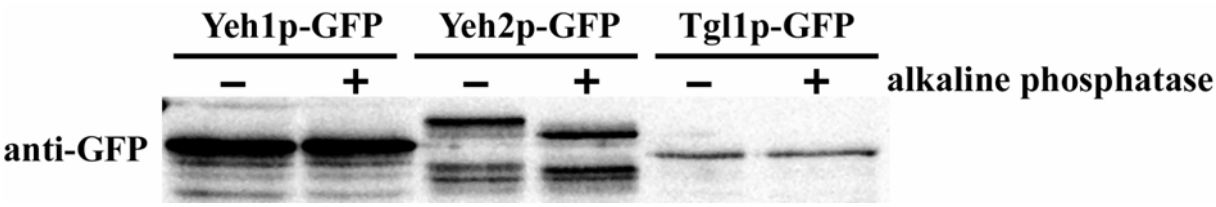

Figure 1: Yeh2p-GFP, but not Yeh1p-GFP or Tgl1p-GFP is phosphorylated. Wild-type strains expressing GFP-tagged lipase Yeh1p (YRS1974), Yeh2p (YRS2086), and Tgl1p (YRS1858) were grown to OD_{600} of 1 in YPD rich media. Microsomal membranes (P13) were prepared as described in (3). For alkaline phosphatase treatment 70 µg microsomal protein pellet was resuspended, divided into two fractions (35 µg protein each), and incubated without or with 2.5 U alkaline phosphatase as described in Materials and Methods. Proteins were precipitated with TCA and resuspended in 35 µl sample buffer. Ten-microgram samples of proteins from untreated and treated fractions were separated by electrophoresis, blotted, and probed with anti-GFP antibody (1:5,000).

Next, we examined the relationship between phosphorylation of Yeh2p and the growth phase. To do so, a preculture of cells expressing Yeh2p-GFP was diluted into fresh media, grown to stationary phase, and protein samples where collected at different time points (Fig. 2A). Western blot analysis of this time course revealed that the none-phosphorylated form of Yeh2p (lower molecular weight form) appears in the logarithmic growth phase and disappears when cells reached high OD_{600} of 8. Thus, Yeh2p seems to be present in the high molecular weight phosphorylated form in cells which enter the early stationary phase (Fig. 2B).

3.2. Identification of the protein kinases responsible for phosphorylation of Yeh2p

To identify the kinases that phosphorylate Yeh2p, 103 strains bearing single deletions of none-essential protein kinases were then tested for altered electrophoretic mobility of Yeh2p-GFP, indicative of a lack of phosphorylation. The protein kinase mutant collection is listed in Table 4. Yeh2p was chromosomally tagged by GFP in all of them and two independent clones of each mutant strain were tested for the electrophoretic mobility of Yeh2p-GFP by western blot analysis. Out of these 103 protein kinase mutants tested, 4 could be identified which showed absence of phosphorylation of Yeh2p. To confirm this result, alkaline phosphatase treatment of protein extracts from the 4 protein kinase deletion strains was performed. As can

be seen in Fig.3, the electrophoretic mobility of Yeh2p-GFP isolated from *kcc4Δ*, *vhs1Δ*, *swe1Δ*, and *ynr047Δ* mutants remains unchanged after dephosphorylation by alkaline phosphatase, indicative of a lack of phosphorylation of Yeh2p in these protein kinase deletion strains. The four protein kinases Kcc4p, Vhs1p, Swe1p, and Ynr047p thus seem to be required for the phosphorylation of Yeh2p. Another interesting result of this screen was that we identified 10 protein kinase mutants which show no expression of Yeh2p-GFP at all, which suggests that certain protein kinases are important for the expression of the STE hydrolase Yeh2p. A summary of the 10 protein kinases which are necessary for the transcriptional regulation of the lipase is shown in Table 5.

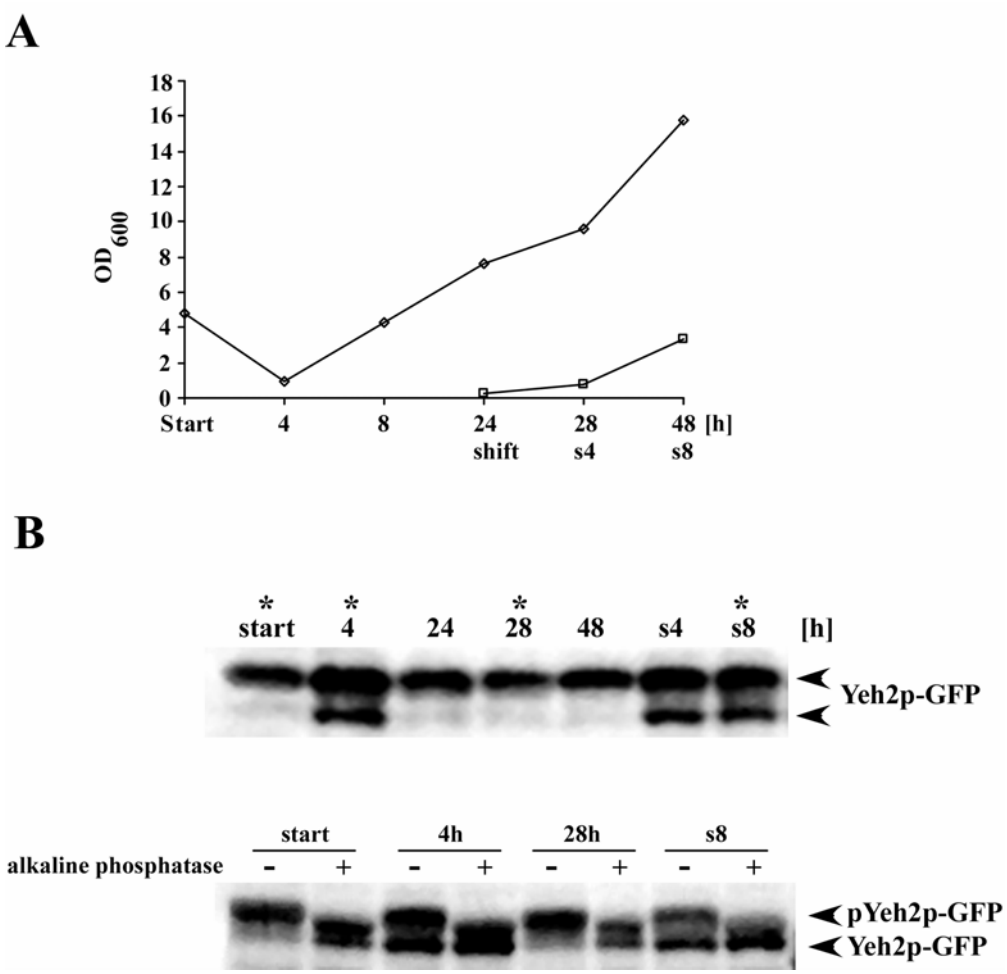

Figure 2: Growth dependent phosphorylation of the lipase Yeh2p. (A) Wild-type expressing a chromosomally tagged version of Yeh2p-GFP (YRS2086) was grown in YPD at 24°C and at indicated time points aliquots equivalent of 2 OD_{600} were removed for western blot analysis. In addition, after 24 h an aliquot was diluted into fresh media and further grown for 8 h to take samples from time point s4 and s8. (B) Total cell lysates were prepared from cells collected in (A) and proteins (equivalent of 0.5 OD_{600}) were separated by SDS-PAGE, followed by immunoblotting with anti-GFP antibody.

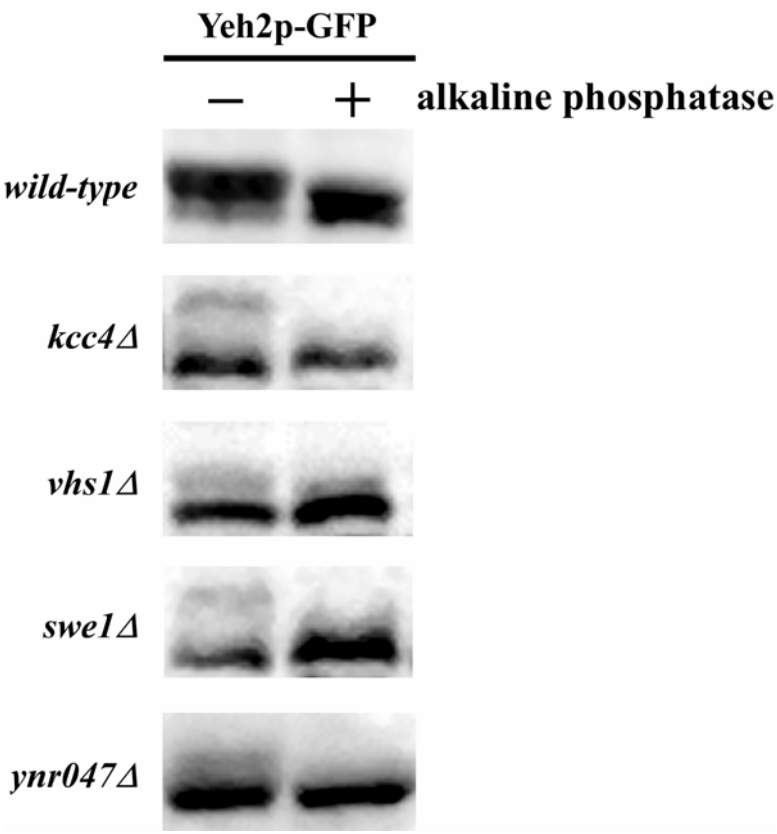

Figure 3: The protein kinases Kcc4p, Vhs1p, Swe1p, and Ynr047p are required for phosphorylation of Yeh2p. Wild-type and protein kinase deletion mutant cells expressing Yeh2p-GFP (wild-type, YRS2086), *kcc4Δ* (YRS2499), *vhs1Δ* (YRS2497), *swe1Δ* (YRS2500), and *ynr047Δ* (YRS2498) were grown in YPD rich media for 16 h. Equivalents of 2 OD_{600} were harvested, divided into two fractions, and treated without (-) or with (+) 2.5 U alkaline phosphatase as described in Materials and Methods. Samples of protein equivalent of 0.5 OD_{600} from each fraction were separated by electrophoresis, blotted, and probed with an anti-GFP antibody.

3.3. Subcellular localization of none-phosphorylated Yeh2p-GFP in protein kinase mutants *kcc4Δ*, *vhs1Δ*, *swe1Δ*, and *ynr047Δ*

The identification of four protein kinases, encoded by *KCC4*, *VHS1*, *SWE1*, and *YNR047*, which are required for phosphorylation of Yeh2p, raised the question about the physiological relevance of the posttranslational modification of the lipase. In mammalians it was shown that phosphorylation of the hormone-sensitive lipase (HSL) does not only lead to activation of the enzyme but also leads to its relocation from the cytoplasm to lipid droplets (1, 2). So we asked the question whether lack of phosphorylation of the yeast STE hydrolase Yeh2p leads to mislocalization of this lipase within the cells. Recent studies showed that Yeh2p-GFP localizes to the plasma membrane and so we investigated the intracellular localization of Yeh2p-GFP in the four protein kinase deletion strains *kcc4Δ*, *vhs1Δ*, *swe1Δ*, and *ynr047Δ* (3). However, microscopic examination of the four mutant strains showed no significant differences in the intracellular localization of Yeh2p-GFP as compared to wild-type cells (Fig.4). The none-phosphorylated Yeh2p-GFP in the protein kinase mutants is found primarily at the plasma membrane and in the vacuole, a localization which is consistent with

our recent results observed in a wild-type strain (3). This suggests that phosphorylation of Yeh2p is not important for proper localization of the lipase in yeast.

Table 5: Protein kinase deletion strains with no detectable expression of Yeh2p-GFP

ORF	Description*
YBR028c	Protein of unknown function localised to cytoplasm
CHK1	regulates inhibitory Cdk phosphorylation of Pds1
MRK1	ser/thr protein kinase
SNF1	carbon catabolite derepressing ser/thr protein kinase
PAK1	DNA polymerase alpha suppressing protein kinase
BUB1	ser/thr protein kinase
HAL5	ser/thr protein kinase
MLP1	ser/thr protein kinase, involved in protection against oxidative stress
KKQ8	ser/thr protein kinase
CLA4	ser/thr protein kinase

*description obtained from MIPS (http://www.mips.gsf.de)

4. Discussion

4.1. The STE hydrolase Yeh2p is posttranslational modified by phosphorylation

The aim of this project was to begin to understand the regulation of the three STE hydrolases Yeh1p, Yeh2p and Tgl1p in yeast and to reveal a possible posttranslational modification (3). Mammalian lipases are often phosphorylated upon hormonal / lipolytic stimuli, which is part of their regulation (1, 2). Given that the yeast STE hydrolases are orthologues of the mammalian lipase family and intrigued by the fact that Yeh2p showed multiple forms of slightly different molecular weight by western blot analysis we examined a possible posttranslational modification of the three yeast lipases. None of the three STE hydrolases is glycosylated (3), however, according to the phosphorylation predicting algorithm *NetPhos2* (http://www.cbs.dtu.dk/services/NetPhos/), Yeh1p, Yeh2p and Tgl1p bear numerous potential phosphorylation sites. A simple test to determine if a protein is phosphorylated or not, is to dephosphorylate the protein by treatment with alkaline phosphatase, which removes the phosphate groups (6). As the dephosphorylated form of a protein sometimes has an altered electrophoretic mobility, the differences between phosphorylated and none-phosphorylated protein can be revealed by western blot analysis. This simple test revealed that Yeh2p, but not Yeh1p and Tgl1p, is phosphorylated (Fig.1).

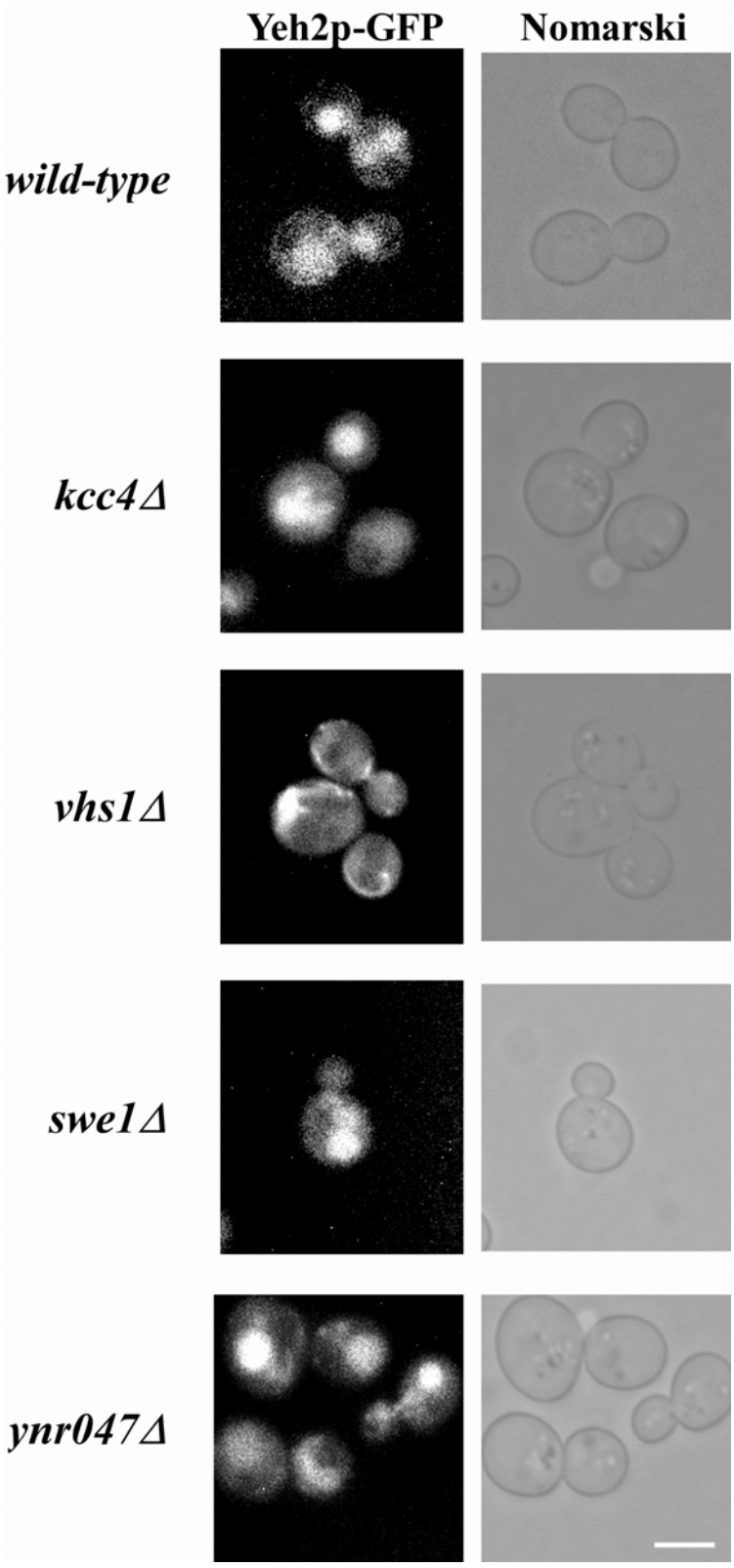

Figure 4: In vivo localization of none-phosphorylated Yeh2p-GFP in *kcc4Δ*, *vhs1Δ*, *swe1Δ*, and *ynr047Δ* protein kinase mutants. Wild-type and protein kinase deletion mutant cells expressing chromosomally tagged version of Yeh2p-GFP (wild-type, YRS2086), *kcc4Δ* (YRS2499), *vhs1Δ* (YRS2497), *swe1Δ* (YRS2500), and *ynr047Δ* (YRS2498) were grown in YPD medium at 24°C and examined by fluorescence microscopy. Bar, 5 µm.

Furthermore, examining the phosphorylation status of Yeh2p as a function of the growth phase indicated that the lipase seems to be phosphorylated in the stationary phase of growth (Fig.2). Dilution of cells into fresh media and restart of growth leads to the rapid appearance of none-phosphorylated or dephophorylated Yeh2p. The biological significance of the phosphorylation of Yeh2p is currently unknown. But activation and inactivation of enzymes by phosphorylation at stationary phase and/or dephosphorylation by a phosphatase is a well known regulatory system in all kingdoms of life. At this point one can only speculate for what reason the steryl ester hydrolase Yeh2p is phosphorylated. There are many examples that phosphorylation by a protein kinase can lead to activation of a lipase, like it is the case for the HSL (1, 2). From this point of view one could suggest that phosphorylation activates the STE hydrolase Yeh2p in yeast. On the other hand, the reverse scenario, that phosphorylation leads to inactivation of the lipase could also be true. However, preliminary experiments in which we examined the *in vitro* STE hydrolase activity of Yeh2p in the plasma membrane of the 4 protein kinase mutant strains showed no significant decrease in the activity of the lipase compared to the lipase in wild-type cells (data not shown). This of course has to be investigated in more detail since one cannot rule out partial redundancy of protein kinases and there is also currently no hint at which degree and at which position phosphorylation is necessary for the activation or deactivation of the lipase. It has also to be mentioned, that phosphorylation possibly affects or changes intracellular localization of a protein. The mammalian HSL is an example where phosphorylation leads not only to activation of the lipase but also to its relocalization to lipid droplets (1, 2). Since the localization studies of Yeh2p-GFP in the protein kinase mutants did not show any alterations in the localization of the lipase as compared to wild-type (Fig.4), it seems unlikely that phosphorylation of Yeh2p would regulate its subcellular distribution.

4.2. The protein kinase mutant screen

In order to identify the protein kinase (s) which phosphorylate Yeh2p we the screened the whole collection of 103 none-essential yeast protein kinase single deletion strains for candidate gene which is necessary for the phosphorylation of Yeh2p. We took advantage of the fact that the none-phosphorylated form of Yeh2p migrates much faster than the phosphorylated form. This allowed us, after chromosomal tagging of Yeh2p by GFP in every single deletion protein kinase mutant strain, to quickly analyze the electrophoretic mobility of Yeh2p-GFP in protein kinase mutants and to compare it to wild-type by western blot analysis. One hundred and three protein kinase mutants were tested this way and in the end we identified 4 protein kinases mutants, *KCC4, VHS1, SWE1*, and *YNR047*, which are required

for phosphorylation of Yeh2p. This result was confirmed by dephosphorylation of Yeh2p-GFP in the kinase mutant strains by treatment with alkaline phosphatase (Fig.3). The electrophoretic mobility of Yeh2p-GFP in the protein kinase mutants does not change upon treatment with alkaline phosphatase indicating that the protein is present in a none-phosphorylated form. Furthermore, Yeh2p-GFP shows a single low molecular weight band which has the same size as the alkaline phosphatase treated form of Yeh2p-GFP from wild-type cells. These results thus indicate that the 4 protein kinases identified in the screen seem to be crucial for the phosphorylation of Yeh2p. Unfortunately, little information about the precise role of the 4 protein kinases can be found in the literature. *KCC4* is a protein kinase of the bud neck involved in the septin checkpoint and it associates with septins. Kcc4p negatively regulates Swe1p by phosphorylation and shows structural homology to the bud neck kinases Gin4p and Hsl1p (7). *VHS1* is a cytoplasmic serine/threonine protein kinase and was identified as a high-copy suppressor of the synthetic lethality of a *sis2 sit4* double mutant, suggesting a role in G1/S phase progression (8). *SWE1* encodes for a protein kinase that regulates the G2/M transition by inhibition of the Cdc28p kinase activity. It localizes to the nucleus and to the daughter side of the mother-bud neck and is a homolog of the *S. pombe* Wee1p. Furthermore, Swe1p seems to be a potential Cdc28p substrate (9). *YNR047w* is a putative protein kinase that, when overexpressed, interferes with pheromone-induced growth arrest. It localizes to the cytoplasm and is also a potential Cdc28p substrate (9). Screening 103 protein kinase mutants did not only help to identify 4 protein kinases required for phosphorylation of Yeh2p, but it also led to the identification of 10 protein kinase mutants in which Yeh2p is not expressed at all (Table 5). This finding is also of great value since protein kinases play important role in regulation of environmental responses, cell cycle and intracellular processes on the level of transcription of important gene products. It could be that the none-expressing protein kinase mutants play an important role in transcription not only of Yeh2p, but maybe also of other lipolytic enzymes in yeast. Therefore it is necessary also to test the expression of the other two lipases Yeh1p and Tgl1p in these mutant strains.

Taken together, we have begun to characterize posttranslational modifications of Yeh2p in the form of phosphorylation which should help us to start to understand the regulation of the activity of STE hydrolases in yeast. Phosphorylation as a posttranslational modification of lipolytic enzymes in yeast is likely to become important because it has been suggested recently that Tgl4p and Tgl5p two yeast triacylglycerol lipases involved in TAG mobilization are potential Cdc28p substrates (9,10). Screening of the none-essential protein kinases in yeast for their requirement for phosphorylation of Yeh2p revealed 4 candidate kinases Kcc4p,

Vhs1p, Swe1p and Ynr047p, required for the phosphorylation of the lipase. The biological significance of the protein kinases in terms of phosphorylation of Yeh2p is presently unclear but is on its way to be characterized in more detail. The investigations summarized here provide a good starting point for further research in the field of regulation of lipolytic enzymes, especially STE hydrolases, in yeast.

Table 3: Essential protein kinases of *Saccharomyces cerevisiae*

#	*ORF*	*name*	Description / Function*
1	*YBL105c*	*PKC1*	ser/thr protein kinase
2	*YBR160w*	*CDC28*	cyclin-dependent protein kinase
3	*YDL017w*	*CDC7*	protein kinase
4	*YDL028c*	*MPS1*	serine/threonine/tyrosine protein kinase
5	*YDL108w*	*KIN28*	cyclin-dependent ser/thr protein kinase
6	*YFL029c*	*CAK1*	cdk-activating protein kinase
7	*YMR001c*	*CDC5*	protein kinase, involved in regulation of DNA replication
8	*YNL161w*	*CBK1*	Protein kinase involved in cell wall biosynthesis
9	*YPL153c*	*RAD53*	ser/thr/tyr protein kinase
10	*YPL204w*	*HRR25*	casein kinase I, ser/thr/tyr protein kinase
11	*YPL209c*	*IPL1*	ser/thr protein kinase
12	*YPR161c*	*SGV1*	ser/thr protein kinase
13	*YAR019c*	*CDC15*	protein kinase of the MAP kinase kinase kinase family

Table 4: None-essential protein kinases of *Saccharomyces cerevisiae*

#	*ORF*	*name*	Description / Function*
1	*YAL017w*	*PSK1*	PAS kinase involved in the control of sugar metabolism and translation
2	*YAR018c*	*KIN3*	ser/thr protein kinase
3	*YBL016w*	*FUS3*	mitogen-activated protein kinase (MAP kinase)
4	*YBR028c*		Protein of unknown function localised to cytoplasm
5	*YBR059c*	*AKL1*	Ark-family Kinase-Like protein
6	*YBR097w*	*VPS15*	ser/thr protein kinase
7	*YBR274w*	*CHK1*	regulates inhibitory Cdk phosphorylation of Pds1
8	*YCL024w*	*KCC4*	coordinates cell cycle progression with the organization of the peripheral cytoskeleton
9	*YCR008w*	*SAT4*	serine/threonine-specific protein kinase
10	*YCR073c*	*SSK22*	MAP kinase kinase kinase
11	*YCR091w*	*KIN82*	ser/thr protein kinase
12	*YDL025c*		putative protein kinase
13	*YDL079c*	*MRK1*	ser/thr protein kinase
14	*YDL101c*	*DUN1*	protein kinase
15	*YDL159w*	*STE7*	ser/thr/tyr protein kinase of MAP kinase kinase family
16	*YDL214c*	*PRR2*	possible role in MAP kinase signaling in the pheromone response pathway
17	*YDR122w*	*KIN1*	ser/thr protein kinase
18	*YDR247w*	*VHS1*	protein kinase involved in G1/S transition
19	*YDR283c*	*GCN2*	ser/thr protein kinase
20	*YDR466w*	*PKH3*	Protein kinase activator of Pkc1-mitogen-activated protein kinase pathway
21	*YDR477w*	*SNF1*	carbon catabolite derepressing ser/thr protein kinase
22	*YDR490c*	*PKH1*	ser/thr protein kinases
23	*YDR507c*	*GIN4*	ser/thr protein kinase
24	*YDR523c*	*SPS1*	ser/thr protein kinase
25	*YER123w*	*YCK3*	casein kinase, isoform 3
26	*YER129w*	*PAK1*	DNA polymerase alpha suppressing protein kinase
27	*YFL033c*	*RIM15*	protein kinase involved in expression of meiotic genes
28	*YFR014c*	*CMK1*	Ca2+/calmodulin-dependent ser/thr protein kinase type I
29	*YGL019w*	*CKB1*	casein kinase II, beta subunit
30	*YGL158w*	*RCK1*	ser/thr protein kinase
31	*YGL179c*	*TOS3*	related to and redundant with Elm1p and Pak1p in activating the SNF1 complex
32	*YGL180w*	*ATG1*	essential for autophagocytosis
33	*YGR040w*	*KSS1*	ser/thr protein kinase of the MAP kinase family
34	*YGR052w*	*FMP48*	ser/thr protein kinase, mitochondrial
35	*YGR080w*	*TWF1*	twinfilin, an actin monomer sequestering protein
36	*YGR092w*	*DBF2*	ser/thr protein kinase related to Dbf20p
37	*YGR188c*	*BUB1*	ser/thr protein kinase

Table 4 continue: None-essential protein kinases of *Saccharomyces cerevisiae*

#	ORF	name	Description / Function*
38	*YGR262c*	*BUD32*	Protein involved in bud-site selection, putative O-sialoglyco-endopeptidase
39	*YHL007c*	*STE20*	ser/thr protein kinase of the pheromone pathway
40	*YHR030c*	*SLT2*	ser/thr protein kinase of MAP kinase family
41	*YHR079c*	*IRE1*	protein kinase
42	*YHR082c*	*KSP1*	ser/thr protein kinase
43	*YHR102w*	*KIC1*	ser/thr protein kinase that interacts with Cdc31p
44	*YHR135c*	*YCK1*	casein kinase I isoform
45	*YHR205w*	*SCH9*	ser/thr protein kinase, stress response and nutrient-sensing signaling pathway
46	*YIL035c*	*CKA1*	casein kinase II, catalytic alpha chain
47	*YIL095w*	*PRK1*	ser/thr protein kinase involved in regulation of actin cytoskeleton organization
48	*YJL095w*	*BCK1*	ser/thr protein kinase of the MEKK family
49	*YJL106w*	*IME2*	ser/thr protein kinase
50	*YJL128c*	*PBS2*	tyrosine protein kinase of the MAP kinase kinase family
51	*YJL141c*	*YAK1*	ser/thr protein kinase
52	*YJL164c*	*TPK1*	cAMP-dependent protein kinase 1, catalytic chain
53	*YJL165c*	*HAL5*	ser/thr protein kinase
54	*YJL187c*	*SWE1*	ser/tyr dual-specifity protein kinase
55	*YJR059w*	*PTK2*	involved in polyamine uptake
56	*YKL048c*	*ELM1*	ser/thr-specific protein kinase
57	*YKL101w*	*HSL1*	ser/thr protein kinase, coupling septin ring assembly to cell cycle progression
58	*YKL116c*	*PRR1*	possible role in MAP kinase signaling in the pheromone response pathway
59	*YKL126w*	*YPK1*	ser/thr-specific protein kinase
60	*YKL139w*	*CTK1*	carboxy-terminal domain (CTD) kinase, alpha subunit
61	*YKL161c*	*(MLP1)*	ser/thr-specific protein kinase, involved in protection against oxidative stress
62	*YKL166c*	*TPK3*	cAMP-dependent protein kinase 3, catalytic chain
63	*YKL168c*	*KKQ8*	ser/thr protein kinase
64	*YKL171w*		weak similarity to ser/thr protein kinase
65	*YKL198c*	*PTK1*	polyamine transport enhancing protein
66	*YLL019c*	*KNS1*	ser/thr protein kinase
67	*YLR096w*	*KIN2*	ser/thr protein kinase
68	*YLR113w*	*HOG1*	ser/thr protein kinase of MAP kinase (MAPK) family
69	*YLR248w*	*RCK2*	Ca/calmodulin-dependent ser/thr protein kinase
70	*YLR362w*	*STE11*	ser/thr protein kinase of the MEKK family
71	*YMR104c*	*YPK2*	ser/thr protein kinase
72	*YMR139w*	*RIM11*	ser/thr protein kinase
73	*YMR216c*	*SKY1*	Protein serine kinase
74	*YMR291w*		putative to ser/thr protein kinase
75	*YNL020c*	*ARK1*	Actin Regulating Kinase

Table 4 continue: None-essential protein kinases of *Saccharomyces cerevisiae*

#	ORF	name	Description / Function*
76	*YNL154c*	*YCK2*	casein kinase I isoform
77	*YNL183c*	*NPR1*	ser/thr protein kinase
78	*YNL298w*	*CLA4*	ser/thr protein kinase
79	*YNL307c*	*MCK1*	ser/thr/tyr protein kinase
80	*YNR031c*	*SSK2*	MAP kinase kinase kinase of the high osmolarity signal transduction pathway
81	*YNR047w*		ser/thr protein kinase, pheromone-response regulator
82	*YOL016c*	*CMK2*	Ca2+/calmodulin-dependent ser/thr protein kinase, type II
83	*YOL045w*	*PSK2*	PAS domain-containing Serine/threonine Kinase
84	*YOL100w*	*PKH2*	ser/thr protein kinase
85	*YOL113w*	*SKM1*	Ste20/PAK-like protein kinase
86	*YOL128c*	*YGK3*	protein activates transcription of stress responsive genes
87	*YOR039w*	*CKB2*	casein kinase II beta chain
88	*YOR061w*	*CKA2*	casein kinase II alpha chain
89	*YOR231w*	*MKK1*	ser/thr protein kinase
90	*YOR233w*	*KIN4*	ser/thr protein kinase
91	*YOR267c*	*HRK1*	Protein kinase with a role in ion homeostasis
92	*YOR351c*	*MEK1*	ser/thr protein kinase
93	*YPL026c*	*SKS1*	suppressor kinase of snf3
94	*YPL031c*	*PHO85*	cyclin-dependent protein kinase
95	*YPL042c*	*SSN3*	cyclin-dependent CTD kinase
96	*YPL140c*	*MKK2*	protein kinase of the map kinase kinase (MEK) family
97	*YPL141c*		putative protein kinase
98	*YPL150w*		putative ser/thr protein kinase
99	*YPL203w*	*TPK2*	cAMP-dependent protein kinase 2, catalytic chain
100	*YPL236c*		protein kinase localised to vacuolar membrane
101	*YPR054w*	*SMK1*	sporulation-specific MAP kinase
102	*YPR106w*	*ISR1*	protein kinase
103	*YPR111w*	*DBF20*	cell cycle protein kinase related to Dbf2p

*description obtained from MIPS (http://www.mips.gsf.de)

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CHAPTER V

Biogenesis of lipid particles

1. Introduction

Many organisms store neutral lipids in lipid particles or lipid droplets. Lipid particles are important as a storage organelle for energy and in lipid homeostasis from prokaryotes to mammals. This storage organelle consists of a hydrophobic core composed of neutral lipids and is surrounded by phospholipids (1). Apart from the lipid storage function of lipid particles, a set of proteins is found to be associated with this organelle. In yeast, this set of protein includes lipolytic enzymes and proteins required for lipid metabolism (Table 3). However, only few studies that address basic mechanisms of lipid particle formation are available. While the exact mechanism of lipid particle biogenesis is still unclear several hypothesis based on recent research have been proposed (for review see 1, 2, 3). A proposed model of lipid particle biogenesis is shown in Fig. 1. The model suggests that neutral lipids, e.g. STE and TAG, are deposited between the two leaflets of the ER membrane during their synthesis, which also takes place in the ER. Consequently as the amount of neutral lipids increases a droplet / particle emerges which then pinches off the ER and is released into the cytoplasma. Proteins associated with lipid particles in yeast are thought to be already inserted into the ER membrane at the place where the nascent lipid particle pinches off the ER and thus are permanent residents of the growing lipid particle. Recently, we identified two STE hydrolases in yeast, Yeh1p and Tgl1p, which are associated with lipid particles (4). Lipid particle localization of these two lipases was used for preliminary studies to investigate the mechanisms of lipid particle biogenesis in yeast.

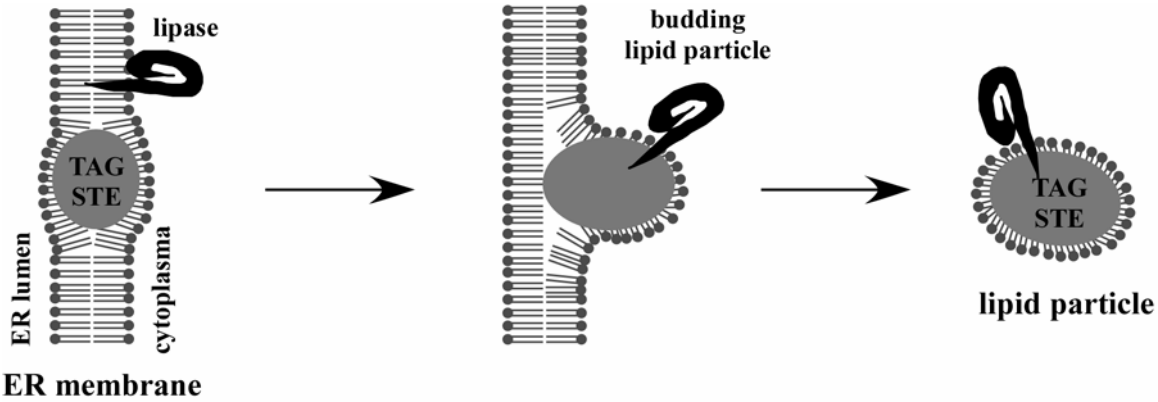

Figure 1: Model of lipid particle biogenesis. Neutral lipids are synthesized in the ER and deposited between the two leaflets of the ER membrane. As the amount of neutral lipids increases the lipid particle emerges, pinches off the ER, and is released into the cytoplasma. Proteins localized to lipid particles are thought to be inserted into the ER membrane at the place where the nascent lipid particle pinches off the ER. STE, steryl ester; TAG, triacylglycerol; (Adapted from Ref. 1).

2. Materials and Methods

2.1. Strains, media, and growth conditions

Yeast strains used in this study are listed in Table 1. Strains were cultivated in YPD rich media (1% Bacto yeast extract, 2% Bacto peptone (USBiological Swampscott, MA), 2% glucose) or YPGal 1% Bacto yeast extract, 2% Bacto peptone (USBiological Swampscott, MA), 2% galactose). Selection for the kanMX4 marker was on media containing 200 µg/ml G418 (Gibco BRL, Life Technologies). Yeast was transformed by lithium acetate (5).

2.2. Chromosomal GFP tagging of Yeh1p and Tgl1p

For C-terminal tagging with GFP, the GFP-HIS3MX6 fusion cassette from pFA6a-GFP(S65T)-HIS3MX6 was amplified by PCR for *YEH1* using primers 5'-ACAGAGGTGGAAACGGAGCTGGAAATGGTTGCTGAGAAGCGGATCCCCGGGTTAATTAA-3' (forward) and 5'-AAATGTATCGTTATGCACGGTGATGTCCCAACTCCCGACAGAATTCGAGCTCGTTTAAAC-3' (reverse) and for *TGL1* using primers 5`-CGACAACTAGATGCCAACTCTTCGACAACTGCGCTGGATCGGATCCCCGGGTTAATTAA-3' and 5`-GATATTAAGACTTCTTATGAAATCCATTTATTGTGTATAGAATTCGAGCTCGTTTAAAC-3'. For N-terminal GFP tagging of *TGL1* a HIS3MX6-PGAL1-GFP fusion cassette from pFA6a-HIS3MX6-PGAL1-GFP(S65T) was amplified by PCR using the primer pair 5`- AACAAGGAAAGAAGAAAGAAAACAATTCGAACAAAACTTTGAATTCGAGCTCGTTTAAAC-3` and 5`-ATCTGTCGATAATCTGCCTAAAAAGGGGAAGTATGCTTTGTATAGTTCATCCATGC-3`. The resulting PCR-fragment was transformed into BY4742 or BY4741 wild-type strains and single deletion strains. Correct integration of the fusion cassette was confirmed by colony PCR using a GFP control primer 5'-TTCGGGCATGGCACTCTTGA-3'.

2.3. Fluorescence microscopy

In vivo localization of the green fluorescent protein (GFP)-tagged version of Yeh1p, Tgl1p, and Yeh2p was performed by fluorescence microscopy using a Zeiss Axioplan 2 (Carl Zeiss, Oberkochen, Germany) equipped with an AxioCam CCD camera and AxioVision 3.1 software.

Table 1: *Saccharomyces cerevisiae* strains used in this study

Strain	Relevant genotype	Source or reference
YRS1974	*MATα his3Δ1 leu2Δ0 ura3Δ0 lys2Δ0 YEH1-GFP-HIS3MX6*	This study; 4
YRS1858	*MATα his3Δ1 leu2Δ0 ura3Δ0 lys2Δ0 TGL1-GFP-HIS3MX6*	This study; 4
YRS2486	*MAT? his3Δ1 leu2Δ0 ura3Δ0 are1::kanMX4 are2::kanMX4*	This study
YRS2665	*MAT? his3Δ1 leu2Δ0 ura3Δ0 are1::kanMX are2::kanMX YEH1-GFP-HIS3MX6*	This study
YRS2662	*MAT? his3Δ1 leu2Δ0 ura3Δ0 are1::kanMX4 are2::kanMX4 TGL1-GFP-HIS3MX6*	This study
YRS2980	*MAT? are1::HIS3 are2::LEU2 dga1::KanMX4 lro1::URA3 ADE2 trp1 TRP1-PGal1-GFP-TGL1*	This study
YRS1789	*MATα his3Δ1 leu2Δ0 ura3Δ0 lys2Δ0 tlg2::kanMX4 YEH1-GFP-HIS3MX6*	This study
YRS1790	*MATα his3Δ1 leu2Δ0 ura3Δ0 lys2Δ0 vps1::kanMX4 YEH1-GFP-HIS3MX6*	This study

3. Results

The first question to be answered was whether the two STE hydrolases Yeh1p and Tgl1p are still localized to lipid particles when no STE are present within the cells. To examine this, a double mutant strain was constructed which is deleted for both STE synthases *ARE1* and *ARE2*. This mutant strain still produces lipid particles, but TAG is the only neutral lipid present in these particles. No STE are detectable in such a deletion mutant background and the lipases Yeh1p or Tgl1p were GFP-tagged on their C-terminus. Fluorescent microscopy studies of these strains revealed that Yeh1p-GFP and Tgl1p-GFP remain localized to lipid particles. This is indicated by the characteristic punctuate intracellular distribution of the GFP-tagged lipases, which resembles the pattern seen in wild-type (Fig.2A). This indicates that STE are not necessary to target the STE hydrolases to lipid particles and that Yeh1p-GFP and Tgl1p-GFP may already be associated with the lipid particle during its biogenesis, regardless of its neutral lipid content.

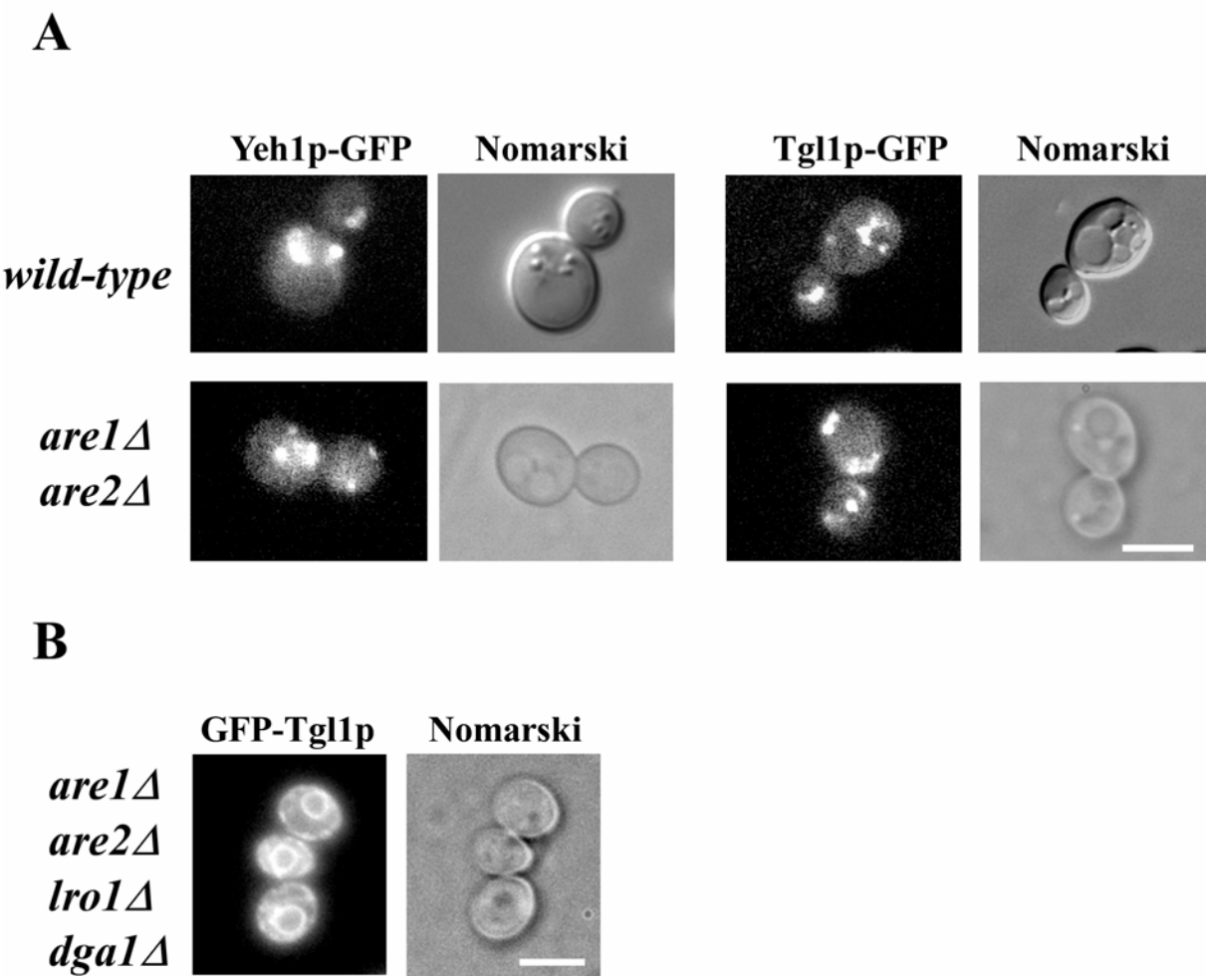

Figure 2: Subcellular localization of Yeh1p and Tgl1p in strains lacking STE and lipid particles. (A) STE hydrolases are associated with lipid particles in a strain defective for STE synthesis. Wild-type and *are1Δ are2Δ* mutant cells expressing C-terminally tagged lipases (YRS1974; Yeh1p-GFP; YRS1858, Tgl1p-GFP; YRS2665, *are1Δ are2Δ* Yeh1p-GFP; YRS2662, *are1Δ are2Δ* Tgl11p-GFP;) were grown in YPD rich medium overnight and examined by fluorescence microscopy. Bar, 5µm. (B) In vivo localization of GFP-Tgl1p in a mutant strain lacking lipid particles. N-terminally GFP-tagged Tgl1p was localized in quadruple mutant cells (YRS2980, *are1Δ are2Δ lro1Δ dga1Δ* GFP-Tgl1p;) after induction in galactose-containing medium for 6 h. Bar, 5µm.

Next, the intracellular localization of the STE hydrolases was examined in a mutant strain which bears virtually no lipid particles at all. This quadruple mutant strain is deleted for the two STE synthesizing enzymes *ARE1* and *ARE2*, as well as for *LRO1* and *DGA1*, enzymes required for the formation of TAG, the second major neutral lipid. For this localization studies Tgl1p was tagged with GFP on the N-terminus and placed under the control of the inducible *GAL1* promotor. Expression of GFP-Tgl1p was induced by growing the cells in galactose containing media. Microscopic analysis clearly showed that GFP-Tgl1p was retained in the ER as there are no lipid particles present (Fig.2B). Even strong overexpression of GFP-Tgl1p

did not change its localization. The STE hydrolase Tgl1p stayed in the ER as indicated by characteristic circular ER staining around the nucleus.

In additional experiments, the localization of the STE hydrolase Yeh1p-GFP was assessed in different mutant strains with defects in the secretory / endocytotic pathway. These strains are single deletion strains for components of the secretory pathway such as *vps* mutants or which bear temperature-sensitive alleles such as *sec7-1*. Temperature-sensitive strains were examined at permissive and restrictive temperature and a complete list of the mutant strains which where tested is summarized in Table 2. However, none of the tested mutants showed aberrant localization of Yeh1p-GFP. Selected examples can be seen in Fig.3. Taken together, lipid particle biogenesis seems to be independent of the secretory / endocytotic pathway. Furthermore, Yeh1p seems to be already associated with lipid particles in the ER and thus it is unlikely that it has to be transported to the site of action via a vesicular route.

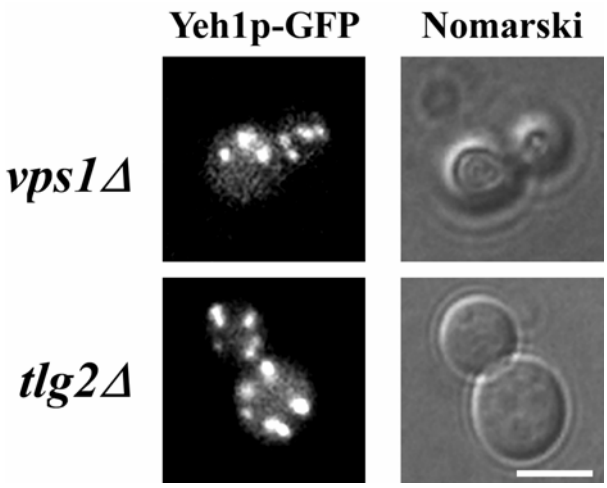

Figure 3: Localization of Yeh1p-GFP in secretory pathway mutants. Deletion mutant cells expressing GFP-tagged Yeh1p (YRS1790, *vps1Δ* Yeh1p-GFP; YRS1789, *tlg2Δ* Yeh1p-GFP;) were grown in YPD rich medium at 24°C and examined by fluorescence microscopy. Bar, 5µm.

4. Discussion

In this study we examined the biogenesis of lipid particles in yeast by following the localization of two GFP-tagged lipid particle proteins, the STE hydrolases Yeh1p and Tgl1p. Our results show that the lipases are localized to lipid particles independent of their neutral lipid content as tested in strains lacking the STE synthases *ARE1* and *ARE2*. Therefore, STE are not necessary for the localization of the STE hydrolases to the lipid particle. Moreover, STE do not provide a signal for localization of Yeh1p and Tgl1p to their intracellular site of action. It is thus likely that, as proposed recently, proteins of lipid particles are already associated with the growing particle at the site of its biogenesis in the ER (1). Microscopic analysis of strains completely devoid of lipid particles reveals that GFP-Tgl1p is found in a

ring-like structure around the nucleus, which is characteristic for the nuclear ER. This is the first time that it was actually shown that a lipid particle localized protein is retained in the ER when lipid particles are not produced. The results support the hypothesis that resident lipid particle proteins might already be associated with the growing particle at the site of lipid particle biosynthesis in the ER. As observed in the quadruple mutant lacking lipid particles, GFP-Tgl1p is retained in the ER. Interestingly, even strong overexpression of the lipase from an inducible *GAL1* promotor is not sufficient to release the protein from the ER. The signal for this apparent effective retention in the ER has not been studied in more detail. In addition, the examination of the localization of GFP-tagged Yeh1p in different strains defective in the endocytotic and secretory pathway lead to the conclusion that the localization of Yeh1p-GFP is independent of known vesicular transport. This supports the view that Yeh1p and also Tgl1p become localized to lipid particles already in the ER and that they are not transported to the lipid particle by vesicular intermediates.

These preliminary studies also show the value of the lipid particles-less model system described here to uncover the biogenesis of lipid particles. By using one of the neutral lipid synthesizing enzymes like Are1p, Are2p, Lro1p, or Dga1p under control of an inducible *GAL1* promotor it should be possible to induce the formation of lipid particles and simultaneously follow the localization of either Yeh1p or Tgl1p tagged with GFP. Such experiments could help to solve the question whether lipid particles indeed bud off from ER membranes.

5. References

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Table 2: Secretory / endocytotic mutants tested for Yeh1p-GFP localization

ORF	Biological process*
VPS4	Vesicle-mediated transport; Phospholipid transport; Protein localization; Nonselective vesicle transport ;
END3	Receptor mediated endocytosis; Nonselective vesicle endocytosis; Actin filament organization;
END4	Cytokinesis; Vesicle-mediated transport; Actin filament organization;
sec7-1	ER to Golgi transport; Protein catabolism; Nonselective vesicle transport; Exocytosis;
sec12-1	ER to Golgi transport; Nonselective vesicle transport; Nuclear pore organization and biogenesis;
SEC18	Secretory pathway; Vesicle-mediated transport; ER to Golgi transport; Nonselective vesicle transport;
SEC6	Vesicle-mediated transport; Nonselective vesicle fusion; Lipid transport; Exocytosis;
VPS34	Golgi to vacuole transport;
PEP12	Receptor mediated endocytosis; Vesicle-mediated transport; Nonselective vesicle fusion;
TLG2	Vesicle-mediated transport
VPS18	Nonselective vesicle fusion; Golgi to vacuole transport; Nonselective vesicle transport;
VPS11	Golgi to vacuole transport; Nonselective vesicle transport;
VPS16	Vesicle-mediated transport; Golgi to vacuole transport; Protein processing; Protein-vacuolar targeting;
VPS33	Golgi to vacuole transport; Protein complex assembly; Vacuolar acidification;
RCY1	Vesicle-mediated transport; Nonselective vesicle transport; Endosome transport;
VPS1	Protein-Golgi targeting; Golgi to vacuole transport; Meiosis; Protein-vacuolar targeting;

* data obtained from YPD (www.proteome.com)

Table 3: Lipid particle proteins in *Saccharomyces cerevisiae*

		Localization		Molecules / cell
		Daum, et al.	*Huh, et al.*	*Ghaemmaghami, et al.*
Name	**Function***	*(1999)*	*(2003)*	*(2003)*
ERG6	Delta(24)-sterol C-methyltransferase, required for ergosterol biosynthesis	LP	LP	53800
ERG7	Lanosterol synthase, required for ergosterol biosynthesis	LP	LP	2190
FAA4	Long-chain fatty acid CoA ligase (for degradation or incorporation into PL	LP	LP	31200
FAT1	Very long-chain fatty acid activator; fatty acid transport protein (exogenous long-chain fatty acids	LP	LP	16900
PET10	Protein of unknown function that co-purifies with lipid particles; expression pattern suggests a role in respiratory growth	LP	LP	2160
TGL3	Triacylglycerol lipase involved in TAG mobilization	LP	LP	3210
AYR1	NADPH-dependent 1-acyl dihydroxyacetone phosphate reductase	LP	ER	3670
EHT1	Putative serine hydrolase	LP	ER	2550
ERG1	Squalene epoxidase, required for ergosterol biosynthesis	LP	ER	65400
FAA1	Long-chain fatty acid CoA ligase (for degradation or incorporation in PL)	LP	ER	7470
SLC1	1-acyl-sn-gylcerol-3-phosphate acyltransferase	LP	ER	technical problems
TDH1	GAPDH	LP	CYT	120000
TDH2				121000
TDH3				169000

Table 3 continue: Lipid particle proteins in *Saccharomyces cerevisiae*

Name	Function*	Localization		Molecules / cell
		Daum, et al.	*Huh, et al.*	*Ghaemmaghami, et al.*
		(1999)	*(2003)*	*(2003)*
YIM1	Protein of unknown function	LP	ER	6540
YJU3	Serine hydrolase with sequence similarity to monoglyceride lipase (MGL)	LP	ER	2140
YOR059c	Hypothetical protein	LP	amb.	1210
YDL193w	Prenyltransferase, required for cell viability	LP	ER	not visualized
TGL1	Steryl ester hydrolase responsible for STE mobilization	LP	p.c.	1470
BSC2	Protein of unknown function	n.d.	LP	922
COY1	Golgi membrane protein with similarity to mammalian CASP	n.d.	LP	2650
ERG27	3-keto sterol reductase, required for ergosterol biosynthesis	n.d.	LP	low signal
PDR16	Phosphatidylinositol transfer protein (PITP) controlled by the multiple drug resistance regulator Pdr1p	n.d.	LP	15400
SNA2	Protein of unknown function, has similarity to Pmp3p, which is involved in cation transport	n.d.	LP	20400
SNX41	Sorting nexin, involved in the retrieval of late-Golgi SNAREs from the post-Golgi endosome to the trans-Golgi network	n.d.	LP	1800
SSO1	Plasma membrane t-SNARE involved in fusion of secretory vesicles at the plasma membrane	n.d.	LP	450
USE1	Essential SNARE protein localized to the ER, involved in retrograde traffic from the Golgi to the ER	n.d.	LP	937
YBR042c	Hypothetical protein	n.d.	LP	2010
YCL005w	Protein of unknown function; null mutant shows a reduced affinity for the alcian blue dye suggesting a decreased net negative charge of the cell surface	n.d.	LP	149

Table 3 continue: Lipid particle proteins in *Saccharomyces cerevisiae*

Name	Function*	Localization		Molecules / cell
		Daum, et al.	*Huh, et al.*	*Ghaemmaghami, et al.*
		(1999)	*(2003)*	*(2003)*
YMR110c	Putative fatty aldehyde dehydrogenase	n.d.	LP	2930
YMR110c	Putative fatty aldehyde dehydrogenase	n.d.	LP	2930
YMR148w	Hypothetical protein	n.d.	LP	922
YOL048c	Hypothetical protein	n.d.	LP	low signal
YOR246c	Protein with similarity to oxidoreductases	n.d.	LP	967
TGL4	Triacylglycerol lipase involved in TAG mobilization	n.d.	LP	195
TGL5	Triacylglycerol lipase involved in TAG mobilization	n.d.	LP	358
YEH1	Steryl ester hydrolase responsible for STE mobilization	n.d.	LP	7770

*Description obtained from SGD (www.yeastgenome.org); LP, lipid particles; ER, endoplasmatic reticulum; CYT, cytoplasma; p.c., punctate composite; amb., ambigious; n.d., not detected

CHAPTER VI

YGR263c/SAY1 encodes for a sterol-acetate deacetylase in Saccharomyces cerevisiae

1. Introduction

To identify lipases required for STE hydrolysis we screened the yeast genome for putative STE hydrolase encoding genes. We were able to find 8 predicted STE hydrolase (Table 2) encoding genes within the genome sequences and tested deletion mutants of these candidate genes for their ability to mobilize STE *in vivo*. This led to the identification of three STE hydrolases in yeast, encoded by *YEH1*, *YEH2*, and *TGL1* (1). However, when we tested deletion mutants of the 8 candidate lipases for mobilization of radiolabeled cholesteryl ester we noticed that in a *ygr263Δ* strain a novel sterol derivate appeared, when extracted lipids were analyzed by TLC. Although mobilization of cholesteryl ester (CE) was not dependent on *YGR263c*, the unknown sterol derivate, which accumulates in the absence of Ygr263p, attracted our attention. Further analysis identified this novel cholesterol derivate as cholesterol acetate (CA). As this work further progressed, Rashi Tiwari was able to show that *ATF2*, encoding for an alcohol-acetylase of yeast, is the enzyme that catalyzes the acetylation of cholesterol (Tiwari *et al.*; manuscript in preparation). Interestingly, sterol-acetate is usually not detected in lipid extracts of yeast, unless the strain is deleted for *YGR263c*. This suggests that Atf2p carries out the acetylation of sterols, especially cholesterol, which then gets deacetylated by Ygr263p. The esterase activity of Ygr263p was confirmed using an *in vitro* assay (Tiwari *et al.*; manuscript in preparation). Thus, *YGR263c* encodes a sterol acetyl deacetylase and was named *SAY1* (Sterol deAcetylase of Yeast 1). The biological meaning of this acetylation – deacetylation cycle of sterols in yeast is still speculative, but there are indications that acetylation of 3β-hydroxysteroids by Atf2p constitutes a detoxification process (2). Since steroids are derived from cholesterol and thus share structural similarity, it is possible that acetylation of sterols, especially cholesterol, could protect cells against the toxic effects of different sterols, such as phytosteroids. This finding, however, is intriguing since excess sterols were thought to be “detoxified” by esterification with long chain fatty acids and subsequent storage in lipid particles.

2. Materials and Methods

2.1. Strains, media, and growth conditions

Yeast strains used in this study are listed in Table 1. Single deletion mutants were obtained from EUROSCARF (www.rz.unifrankfurt.de/FB/fb16/mikro/euroscarf/index.html), and double-mutant strains were generated by gene disruption with PCR deletion cassettes or by crossing of single-mutants (4). C-terminal tagging of genes was performed by homologous recombination with the PCR fusion cassettes derived from PFA6a-GFP-MYC-HIS3MX6 (4).

Table 1: *Saccharomyces cerevisiae* strains used in this study

Strain	Relevant genotype	Source or reference
YRS1533	BY4742; *MATα his3Δ1 leu2Δ0 ura30 lys2Δ0*	EUROSCARF; 3
YRS2550	*MATα his3Δ1 leu2Δ0 ura30 lys2Δ0 say1*::*kanMX4*	EUROSCARF; 3
YRS2551	*MATα his3Δ1 leu2Δ0 ura30 lys2Δ0 atf2*::*kanMX4*	EUROSCARF; 3
pdr5Δ	*MATα his3Δ1 leu2Δ0 ura30 lys2Δ0 pdr5*::*kanMX4*	EUROSCARF; 3
snq2Δ	*MATα his3Δ1 leu2Δ0 ura30 lys2Δ0 snq2*::*kanMX4*	EUROSCARF; 3
YRS2133	*MATα his3Δ1 leu2Δ0 ura30 lys2Δ0 say1*::*HIS3MX6 atf2*::*kanMX4*	This study
YRS2485	*MATα his3Δ1 leu2Δ0 ura30 lys2Δ0 SAY1-Myc-HIS3MX6*	This study
YRS1853	*MATα his3Δ1 leu2Δ0 ura30 lys2Δ0 say1::kanMX4 hem1::LEU2*	This study
YRS1710	BY4742; *MATα his3Δ1 leu2Δ0 ura30 lys2Δ0 hem1::LEU2*	This study

For growth tests on agar plates, fresh overnight cultures were inoculated into YPD (1% Bacto Yeast Extract, 2% Bacto Peptone [U.S. Biological, Swampscott, Mass.], 2% glucose) to OD_{600} of 0.2 and grown to OD_{600} of 1 at 24°C or 30°C. Equivalents of 2 OD_{600} units of cells were harvested, resuspended in 200 µl YPD and serial 10-fold dilutions were spotted on agar plates with and without test compounds. The agar plates were prepared by adding the different compounds from stock solutions to molten agar equilibrated to 50°C. Tested phytochemicals and final concentrations were: eugenol 0.25 mM to 1.8 mM, genistein 1 mM, kampferoel 1mM, naringenin 0.1 mM to 1 mM, resveratrol 0.1 mM to 1 mM, and farnesol 0.1 mg/ml to 10 mg/ml. Concentrations tested for progesterone were 10 µg/ml and 20 µg/ml in SC+ALL agar plates. Cell growth was examined after 3 to 4 days of incubation at 24°C or 30 °C. All experiments were performed in duplicates.

2.2. Lipid raft isolation (OptiPrep™ gradient) and analysis of proteins and lipids

Cells were grown at 24°C in YPD rich medium to log phase, and 85 OD_{600} units were collected, washed once in water, and stored at -20°C for a minimum time of 1 h. The cell pellet was lysed in 2.8 ml of TNE buffer (30 mM Tris-HCl pH 7.5, 120 mM NaCl, 5 mM EDTA) with a protease inhibitor mixture (1 mM PMSF, 2.5 µg/ml leupeptin, and pepstatin, protease inhibitor cocktail Complete™-Roche) by vortexing with glass beads for 10 min at 4°C. The lysate was cleared of unbroken cells and glass beads by centrifugation at 500 × *g* for

7 min. The cleared lysate (2.4 ml) was then incubated with Triton X-100 (1% final concentration) for 30 min on ice. After the detergent extraction, the lysate (1.1 ml) was adjusted to 40% Optiprep by adding 300 µl TXNE (TNE/0.1% TX100) and 2.8 ml of Optiprep solution (Nycomed, Oslo) and overlaid with 5.72 ml of 30% Optiprep in TXNE (TNE/0.1% TX100) and 550 µl of TXNE. The samples were centrifuged at 37,000 rpm for 2.5 h in a TH641 rotor (Sorvall), and 8 fractions of equal volume (1.2 ml) were collected from the top. Fractions from the gradient were precipitated by adding TCA (10% final concentration), washed once with acetone, resuspended in sample buffer and analyzed by SDS/PAGE and Western blotting. Western blots were probed with rabbit antisera against Pma1 (1:10,000), Gas1 (1:5,000, a kind gift from A. Conzelmann, University of Fribourg), Wbp1 (1:1,000), and mouse antisera against the Myc-epitope (1:500).

For analysis of radiolabeled lipids, *hem1Δ* strains were incubated with 0.025 µCi/ml [^{14}C]cholesterol (American Radiolabeled Chemicals Inc, St. Louis, MO) for 16 h at 24°C. Cells were then diluted into liquid YPD media containing 20 µg/ml cholesterol and 0.05 % Tween 80 to OD_{600} of 0.5 and grown for 4 h at 24°C. Cells were harvested, frozen, broken with glass beads, and lysates were loaded onto OptiPrep gradients as described above. Eight fractions of equal volume were collected from the top of the gradient and lipids were extracted 2 times with chloroform/methanol (1:1; vol/vol). Next, the organic phase was washed once with water and extracted once more with chloroform. Extracted lipids were brought to dryness, resuspended in 20 µl chloroform/methanol (1:1; vol/vol), and radioactivity in the lipid extract was determined by scintillation counting. Total lipid extracts were separated on TLC plates (Merck, Darmstadt, Germany) developed in petroleum ether/diethylether/acetic acid (70:30:2; per vol.) and quantified by scanning with a Berthold Tracemaster 40 Automatic TLC-Linear Analyzer. TLC plates were then exposed to a tritium sensitive screen and visualized using a phosphorimager (Bio-Rad Laboratories, Hercules, CA).

3. Results

3.1. Say1p-Myc is not associated with lipid rafts.

In order to determine the subcellular localization of Ygr263p/Say1p in more detail we investigated lipid raft association of Say1p using OptiPrep gradient centrifugation. As can be seen from Fig.1A, Say1p-Myc is absent from the top fractions of the gradient, where lipid raft associated proteins like Pma1p and Gas1p are enriched. Instead, Say1p-Myc is solubilized

from membranes and can be detected at the bottom of the gradient, as is the non-lipid raft marker protein Wbp1p. This indicates that Say1p is not associated with lipid rafts in yeast.

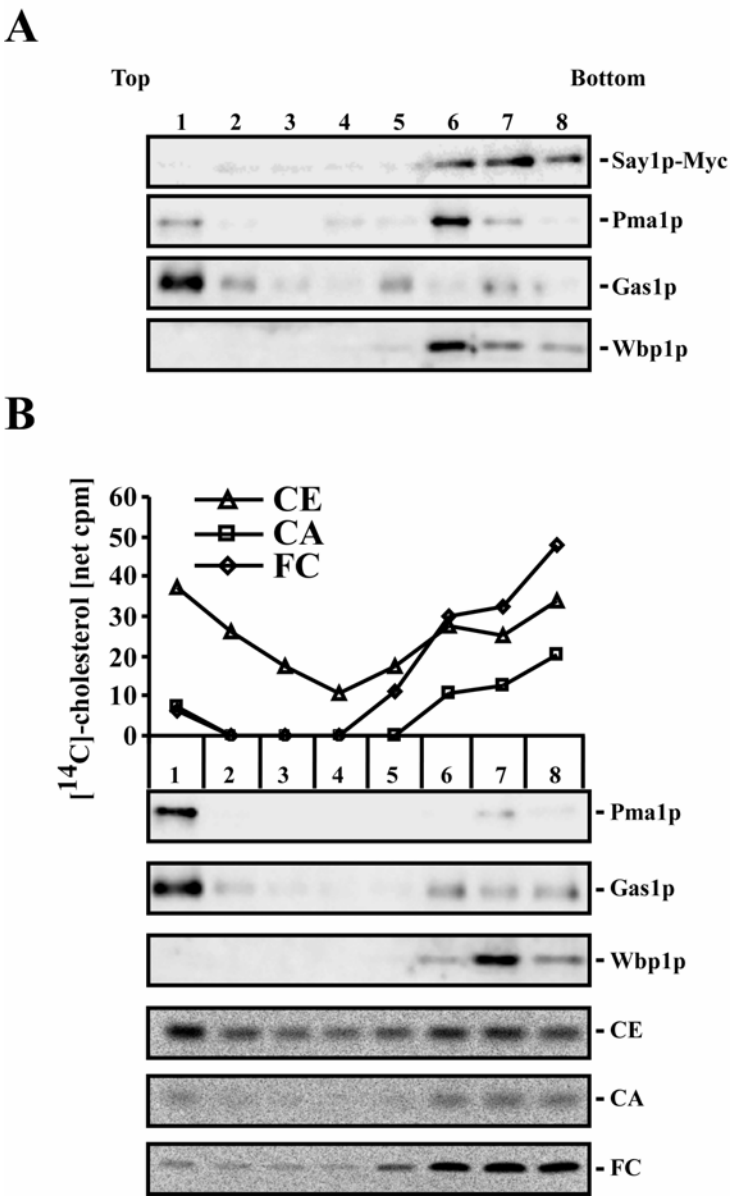

Figure 1: OptiPrep lipid raft isolation. (A) Say1p-Myc is not associated with lipid rafts. A strain expressing chromosomally tagged *ATF2-Myc* (YRS2485) was grown to logarithmic phase and OptiPrep gradient centrifugation of total protein was performed as described under Materials and Methods. Total protein from 8 fractions was precipitated and further analyzed by western blotting using antibodies against Pma1p (1:10,000), Gas1p (1:5,000), Wbp1p (1:1,000), and the Myc-epitope (1:500). (B) Distribution of free cholesterol (FC), cholesterol acetate (CA), and cholesterol ester (CE) in *say1Δ* mutants. Heme-deficient wild-type and single-deletion mutant (*hem1Δ*, YRS1710; *hem1Δ say1Δ*, YRS1853;) were labelled with [^{14}C]cholesterol as described under Materials and Methods. Total lipids were extracted, separated by TLC, and quantified by scanning with a Berthold Tracemaster 40 Automatic TLC-Linear Analyzer. As a control and for western blotting, total proteins of a non-labelled *hem1Δ say1Δ* mutant (YRS1853) were subjected to OptiPrep gradient centrifugation, performed as described in Fig.1A. CE, cholesterol ester; CA, cholesterol acetate; FC, free cholesterol;

Next, we analyzed a *say1Δ* mutant strain for the distribution of free cholesterol (FC), cholesterol acetate (CA), and cholesterol ester (CE) in an OptiPrep gradient. In addition, we also determined if there are any disturbances of lipid raft-association of Pma1p and Gas1p in a mutant strain deleted for *SAY1*. In this mutant strain, we detect both, Pma1p and Gas1p, in the detergent resistant membrane (DRM) fraction at the top of the gradient and indicating that *say1Δ* is not defective in lipid raft-composition and/or lipid raft-association of Gas1p and Pma1p (Fig.1B). Analysis of the distribution of radiolabeled FC, CA and CE in this mutant strain, however, did not reveal clear results. FC and CA is detectable in nearly equal amounts in the top fraction of an OptiPrep gradient, which represents DRM. CE show identical distribution, but in the top fraction CE seem to accumulate. Most likely, this is due to enrichment of lipid particles, which are the intracellular stores of CE, in the top fraction by flotation. The presence of FC in the lipid raft fraction is in agreement with previous results since a main detergent resistant component of lipid rafts is known to be cholesterol, respectively ergosterol (Fig.1B). Furthermore, the detectable amounts of CA in this fraction suggest that the separation of the detergent soluble lipids from the DRM was not stringent enough. Thus, the presence of CA in the top fractions of the gradient likely reflects contaminations of the DRM fraction in this experiment.

3.2. A *say1Δ* mutant is not sensitive to a variety of phytochemicals and progesterone.

Phytochemicals are plant chemicals that have protective properties. Well known phytochemicals are flavonoids which are commonly found in fruit, vegetables, nuts, seeds, flowers, tea, and wine, as well as isoflavonoids of soy to name just a few. This class of natural products possesses antifungal, antiviral and antibacterial activity (5). In addition, phytochemicals can protect human against disease, as many of these compounds have antioxidant and cholesterol lowering activity. Certain members of phytochemicals share structural homology to hormones and exhibit weak estrogenic effects (6). These estrogenic and anti-estrogenic compounds, also called phytoestrogenes, include isoflavonoids and flavonoids. Here we tested several phytochemicals for antifungal activity on strains lacking either Atf2, Say1, or both, to test whether acetylation - deacteylation of xenocompounds by these gene products is important for detoxification. Therefore we tested the growth of *say1Δ*, *atf2Δ*, and *atf2Δ say1Δ* in the presence of DMSO, ethanol and high salt, which did not reveal any growth differences to wild-type (Fig.2A). We then examined growth in the presence of flavonoids like kampferoel, naringenin, resveratrol, and the isoflavonoid genistein. None of the phytochemicals inhibited growth of the mutant strains compared to wild-type (Fig.2B).

Another substance tested was farnesol, which is an isoprenoid alcohol, and has been shown to inhibit growth of *Saccharomyces cerevisiae* (7). However, we could not observe any differences in growth of the tested strains on media containing farnesol (Fig.2B).

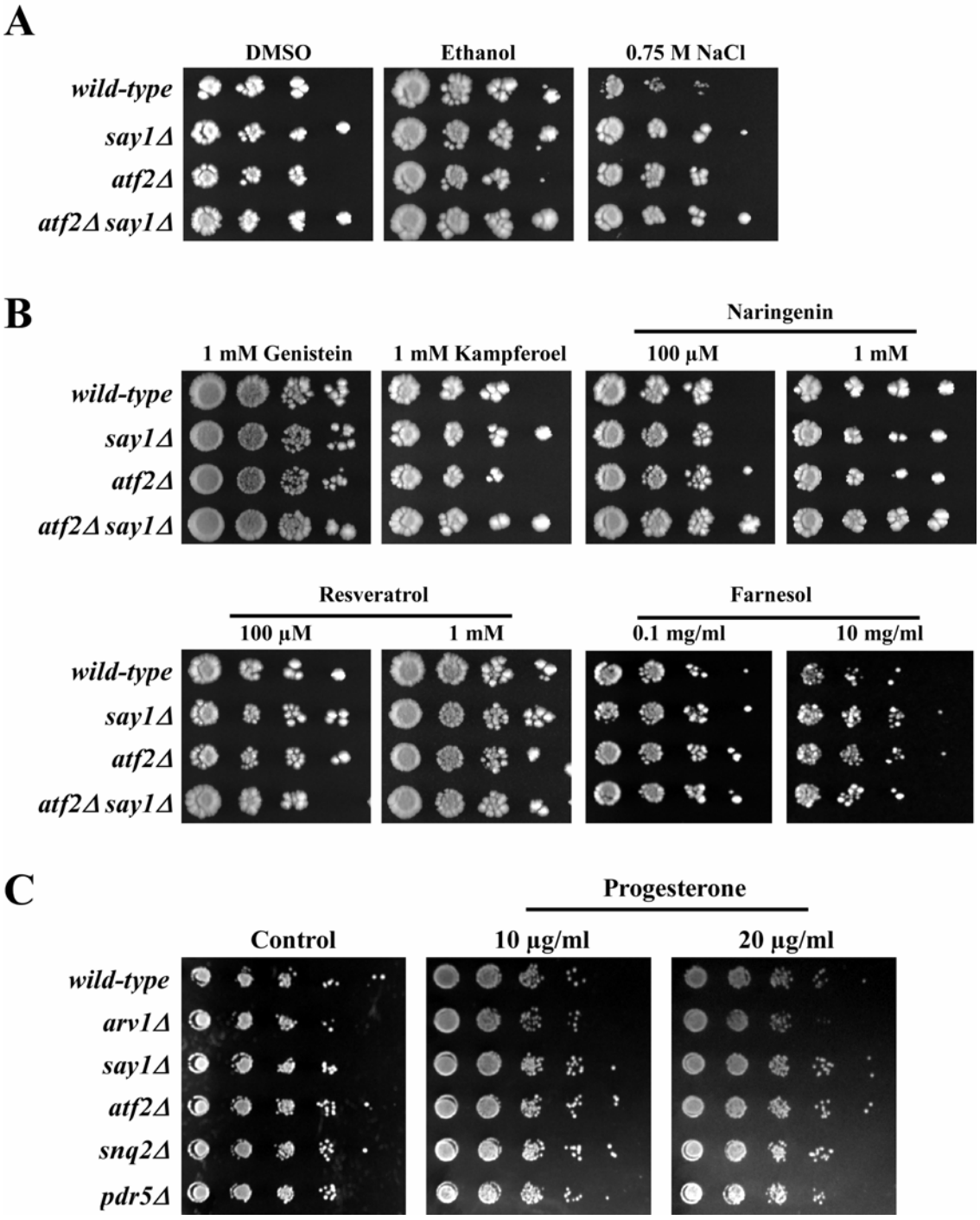

Figure 2: Growth test of *say1Δ*, *atf2Δ*, and *atf2Δ say1Δ* on media supplemented with phytochemicals and progesterone. Wild-type, single- and double-deletion strains (*wild-type*, YRS1533; *say1Δ*, YRS2550; *atf2Δ*, YRS2551; *atf2Δ say1Δ*, YRS2133;) were spotted and grown on media containing (A) DMSO, ethanol, or 0.75 M NaCl; (B) the indicated Phytochemicals; and (C) Progesterone. After 4 to 5 days at 24°C, growth was determined and images were taken.

Last but not least, we also wanted to examine growth inhibitory effects of progesterone, a mammalian steroid hormone which has been shown to be toxic for yeast cells lacking the ABC-transporter Pdr5 (8). No effect of the steroid on the growth of *atf2Δ*, *say1Δ*, and *atf2Δ say1Δ* mutant strains was observed (Fig.2C). Moreover, deletion of two ABC-Transporters *PDR5* and *SNQ2*, which were reported to be essential for detoxification of progesterone, showed no altered sensitivity to this steroid (8). Taken together, we tested several compounds which are hypothetical targets for a potential detoxification process based on acetylation – deacteylation by Atf2p and Say1p, and thus should inhibit growth of strains deleted for *ATF2* or *SAY1*. Unfortunately, none of the compounds tested showed an inhibitory effect.

3.3. A strain lacking either Atf2p or Say1p is sensitive to Eugenol.

Eugenol, which belongs also to the family of phytochemicals, is the major phenolic constituent of clove oil (90% of total oil) and was found to have antifungal activities against *Saccharomyces cerevisiae*. The chemical structure of eugenol which is a phenolic monoterpene is shown in Fig.1A. Treatment of yeast cells with eugenol leads to alterations of both plasma membrane and cell wall (9). Eugenol concentration of 1.8 mM inhibits the growth of yeast, while 3 mM eugenol is fungicidal (9). When we tested the growth of *atf2Δ*, *say1Δ*, and *atf2Δ say1Δ* on media containing eugenol we observed that mutant strains deleted for either one or both genes exhibited increased sensitivity against eugenol at concentrations of 0.9 mM or 1.8 mM (Fig.1B). Next, we examined the effect of another antimycotic drug, nystatin, on growth of our test strains. The antifungal property of nystatin is due to its interaction with ergosterol which leads to impaired membrane function. Strains with altered plasma membrane composition, e.g. having less or structurally altered sterols, are resistant to nystatin (10, 11). Our results show that single deletion of *ATF2* or *SAY1* lead to nystatin resistance which suggests that these mutants have altered composition of their plasma membrane (Fig.1C). Moreover, combination of both nystatin and eugenol results in loss of resistance to nystatin of the deletion mutants (Fig.1C). This indicates that their plasma membrane is probably severely damaged by eugenol. Taken together, a growth inhibitory effect of the phytochemical eugenol on *atf2Δ* and *say1Δ* mutants, as well as a resistance of both mutants against the polyene antibiotic nystatin was observed.

4. Discussion

The identification of a sterol-acetate deacetylase, encoded by *YGR263c/SAY1*, tempted us to examine a possible physiological role for such an enzyme in yeast. The presence of such an

enzymatic activity was unexpected, as we were originally screening for STE hydrolases in yeast. Interestingly, sterol-acetate seems to be formed in wild-type cells but escapes detection unless the sterol-acetate deactylase is deleted. Further experiments identified the alcohol-acetylase encoded by *ATF2* as the enzyme responsible for acetylation of sterols.

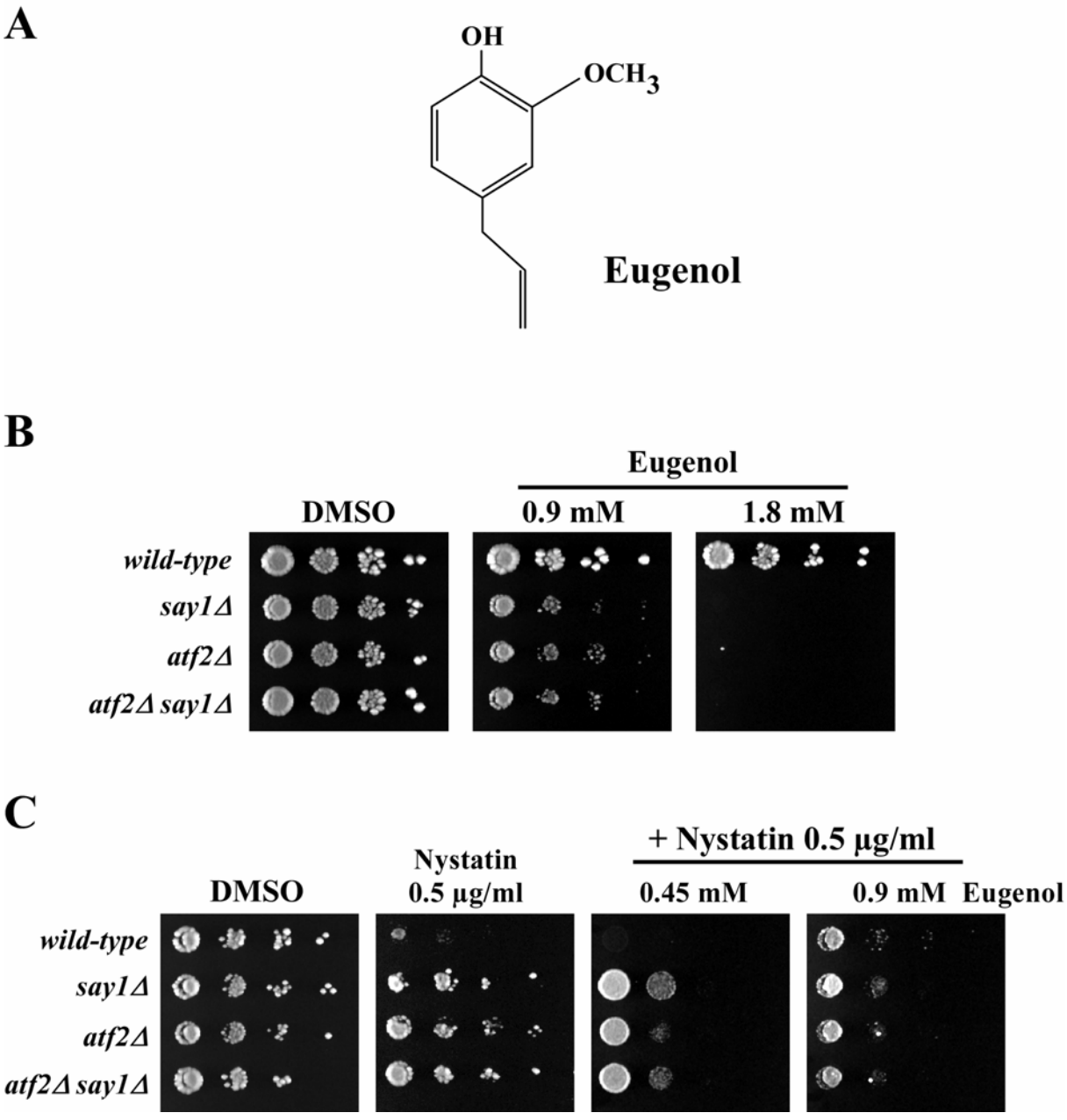

Figure 3: Eugenol inhibits the growth of *say1Δ* and *atf2Δ* strains. (A) Chemical structure of Eugenol. (B) Wild-type, single- and double-deletion strains (*wild-type*, YRS1533; *say1Δ*, YRS2550; *atf2Δ*, YRS2551; *atf2Δ say1Δ*, YRS2133;) were spotted and grown on media containing 0.9 mM and 1.8 mM eugenol; and (C) containing either 0.5 µg/ml nystatin alone or together with 0.45 mM and 0.9 mM eugenol.

This result, together with the determination of the sterol-acetate deacetylase activity of Say1p, suggested that we may have uncovered a novel "cycle" for sterols in yeast (Tiwari *et al.*;

manuscript in preparation). Today the classical view of sterol "cycles" in yeast is that free sterols are esterified with long chain fatty acids to STE, which are then hydrolyzed by STE hydrolases (12, 1). However, this new finding of a sterol acetylation – deactylation cycle, based on the activity of Atf2p and Say1p, raises many additional questions concerning the physiological relevance of this cycle. The observation that Atf2p acetylates 3-beta hydroxysteroids when supplied to yeast, raised speculations that acetylation of "harmful" or "unwanted" compounds constitutes a potential detoxification mechanism (2). The participation of Say1p in a detoxification mechanism, however, remains hypothetical. Say1p could be responsible for "recycling" of substrates which were by default acetylated by Atf2p. This could explain the observation that lipid extracts from wild-type yeast cells contain only very small amounts of sterol-acetate, most of the time even below the detection level. In contrast to this, if the "recycling" enzyme Say1p is missing, an enormous amount of CA accumulates and is easily detectable in lipid extracts as observed in *say1Δ* cells (Tiwari *et al.*; manuscript in preparation). However, we examined the effects of various phytochemicals, as hypothetical substrates for acetylation, on the growth of *atf2Δ*, *say1Δ*, and *atf2Δ say1Δ* mutants. Except for eugenol, none of the 6 tested phytochemicals inhibited growth of the mutant strains. Only for eugenol we obtained an inhibitory effect on growth of our deletion mutant strains. Antifungal activity of eugenol involves alterations of both membrane and the cell wall in yeast (9). Further experiments showed that the single- and double-mutant are also resistant to nystatin treatment. Resistance to nystatin is thought to be caused by alterations of the plasma membrane and thus it can be speculated that the mutant strains tested may have an altered or disturbed plasma membrane. Moreover, this hypothesis is in line with the fact that strains deleted for *ATF2* and/or *SAY1* are sensitive to the membrane and cell wall disturbing phytochemical eugenol. Why strains deleted for either *ATF2* and/or *SAY1* are sensitive to eugenol is not yet clear. Most likely, this sensitivity is due to the fact that *atf2Δ* and/or *say1Δ* mutants have an altered or disturbed plasma membrane and/or cell wall composition due to the missing of one of these two gene products. However, a direct disturbing effect of eugenol on the plasma membrane / cell wall of these mutant cells has also to be taken into account. Mass spectrometry analysis of the plasma membrane / cell wall composition of the mutants treated with eugenol should provide answers for this hypothesis.

Taken together, these results suggest that the deletion mutant strains are affected in their plasma membrane structure/function which makes them sensitive to eugenol, rather than that Atf2p and Say1p are important for detoxification of eugenol. This hypothesis is strengthened

due to the fact that both deletion mutants showed resistance to nystatin, which indicates that the plasma membrane structure/function is altered in *say1Δ* and *atf2Δ* strains.

Table 2: Predicted STE hydrolases in *Saccharomyces cerevisiae*

Name	ORF	Function / Description	Reference
Yeh1	*YLL012w*	Steryl ester hydrolase	1
Yeh2	*YLR020c*	Steryl ester hydrolase	1
Tgl1	*YKL140w*	Steryl ester hydrolase	1
Say1	*YGR263c*	Sterol-acetate deacetylase	Tiwari, *et al.*
Tgl2	*YDR058C*	Protein with lipolytic activity towards triacylglycerols and diacylglycerols when expressed in E. coli	13
	YOR059c	Hypothetical protein, alpha/beta-hydrolase	*
	YJL068c	none-essential intracellular esterase	14
	YLR118c	Acyl-protein thioesterase responsible for depalmitoylation of Gpa1p	15

* http://www.sanger.ac.uk/cgi-bin/Pfam/

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DECLARATION FOR THE FACULTY

I herewith declare that all data and concepts presented in this thesis result from no other sources other than my own work unless stated otherwise.

René Köffel

Klagenfurt, 19th June, 2006

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Köffel R and Schneiter R. (2006). *Yeh1* constitutes the major sterol ester hydrolase under heme-deficient conditions in *Saccharomyces cerevisiae*. *Eukaryot Cell.* ***7:****1018-25*

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Oral Presentations

11/08/2005	"Identification and characterization of a novel family of lipases required for steryl ester hydrolysis in yeast", Workshop 13 XXII. International Conference on Yeast Genetics & Molecular Biology, Bratislava, Slovak Republic
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