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# NEUTRAL LIPID STORAGE IN YEAST

PhD Thesis

by

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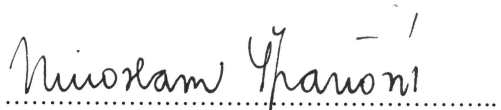

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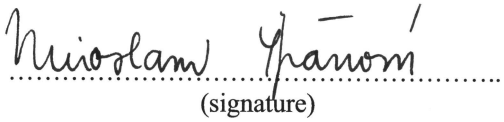

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## Zusammenfassung

Squalen, ein natürliches Triterpen, ist ein wichtiges Zwischenprodukt in der Ergosterol-Biosynthese und kommt unter Standardbedingungen nur in geringer Menge vorkommt. Für die vorliegende Arbeit wurden *Saccharomyces cerevisiae* Stämme konstruiert, die Squalen akkumulieren, um Grundlagenstudien durchzuführen und die Basis für biotechnologische Squalen-Produktion zu schaffen. Zunächst wurde eine Lokalisierung von Squalene in der Zelle vorgenommen und ein möglicher lipotoxischer Effekt untersucht. Es konnte gezeigt werden, dass Squalen auf Grund seiner starken Hydrophobizität in sog. Lipidpartikeln (lipid droplets) lokalisiert ist. In diesen Partikeln stört Squalen die interne Ordnung, die durch Sterolester in Form konzentrischer Schichten um einen Kern aus Triglyceriden entsteht. In Hefezellen, die auf Grund mehrfacher Mutationen keine Lipidpartikel bilden können, übt Squalen keinen lipotoxischen Effekt aus. In solchen Mutanten findet man große Mengen an Squalen in Membranen. Diese Beobachtung war überraschend, denn Squalen ist auf Grund seiner ausschließlich unpolaren Natur kein typisches Membranlipid. Daraus ergab sich die Frage nach den Einflüssen, die Squalen auf die Stabilität von Membranen ausübt. Wir zeigten, dass durch die Gegenwart von Squalen Membranen des Endoplasmatischen Reticulums rigider werden, während die Plasmamembran an Fluidität zunimmt. Der letztgenannte Befund erklärt die Tatsache das Squalen akkumulierende Zellen sensitiv gegen hohen und nieder pH-Wert sowie gegen Detergentien sind. Die Kombination von Ergosterol und Squalen scheint bei diesen Effekten eine wichtige Rolle zu spielen. Zusammenfassend zeigen unsere Resultate dass Squalen (i) sowohl in Lipidpartikeln als auch in Membrane der Hefe abgelagert werden kann, und (ii) trotz seiner für Membranlipide untypischen Eigenschaften als milder Modulator in Membranen fungiert.

## Summary

Squalene, a natural triterpene, is a key intermediate in ergosterol synthesis of the yeast and present in the cell only at minor amounts under standard conditions. For the present study we constructed strains of *Saccharomyces cerevisiae* accumulating squalene to perform fundamental research but also as a possible source for biotechnological production of this lipid. We first addressed localization of squalene within the cell and its possible lipotoxic effect in the yeast. We showed that the highly hydrophobic squalene preferentially localized to lipid particles/droplets. In this compartment it decreases the order created by steryl esters which form concentric layers around a core of triacylglycerols. We also observed that squalene did not exhibit a lipotoxic effect even in mutant strains which are unable to form lipid particles. In strains devoid of lipid particles large amounts of squalene were stored in membranes. This finding was surprising because squalene is not a typical membrane lipid and cannot actively contribute to membrane formation *per se* due to its hydrophobic nature. This observation led us to investigate the influence of squalene on membrane stability. We showed that endoplasmic reticulum membranes became more rigid when enriched with squalene, whereas plasma membrane samples became softer. The latter finding was in line with increased sensitivity of squalene accumulating cells to high and low pH, high salt or detergent concentration. However, the combination of squalene with low or high amounts of sterols in a membrane seems to be important for the squalene effect. In summary, our results demonstrated that squalene (i) can be well accommodated in yeast lipid particles and organelle membranes without causing deleterious effects; and (ii) although not being a typical membrane lipid may be regarded as a mild modulator of biophysical membrane properties.

# General Introduction and Aim of the Study

During the last decades, customers got interested in utilization of various natural products for many purposes. This trend became the basis for discovery and characterization of a large number of components. In the course of these chemical and pharmacological investigations general biological screenings for natural products were performed all over the world. Among the substances which turned out to be of interest there is squalene, an intermediate of sterol biosynthesis. Squalene has become popular for biotechnological research because of its lipophilic properties. It is a natural compound which belongs to the group of isoprenoids and is precursor for the synthesis of sterols, steroids and ubiquinons such as coenzyme Q10. Squalene is one of the most important lipids in skin cells. It affects the normal metabolism of skin and secures beneficial mechanical properties. Squalene is also an antioxidant which neutralizes harmful effects of excessive free radicals produced in the body [1]. Moreover, squalene has been reported to show antilipidemic, cardio-preventive and tumor inhibiting properties [2-4]. Traditionally, squalene has been mostly isolated form shark liver oil. Olive oil and its refining by-product olive oil deodorizer distillate became alternative sources for biotechnological production [5]. Microbial production of squalene is not yet very well studied.

In this Thesis I focused on the biochemistry and cell biology of squalene in the yeast *Saccharomyces cerevisiae*. Under normal growth conditions, squalene does not accumulate in *Saccharomyces cerevisiae*, but absence of oxygen or genetic manipulations such as overexpression of the *HMG1* gene, disruption of *ERG1*, *ERG7* or *HEM1* provide strategies to increase the cellular squalene level.

In the yeast, squalene is an intermediate of ergosterol synthesis. Ergosterol biosynthesis is a highly regulated process, is strictly aerobic and heme-dependent. Heme is necessary for the activity of the sterol-14-α-demethylase Erg11p, an NADPH-heme-dependent P450 protein [6;7]. If heme cannot be formed in *hem1*Δ strains, squalene and lanosterol accumulate [8]. For studies described here we used strains deleted of *HEM1* which encodes the first enzyme in heme synthesis, δ-aminolevulinic acid synthase. Such *hem1*Δ strains have been widely used as a model for anaerobic growth [9;10] because metabolic changes resulting from the lack of cytochromes are similar to oxygen deficiency. As a consequence of manipulations described above, anaerobic yeast and *hem1*Δ cells become auxotrophic for sterols and unsaturated fatty acids [11;12].

Squalene and sterols are mostly synthesized in the endoplasmic reticulum (ER). Sterols are essential building blocks of all membranes. They help maintaining membrane permeability and stability. Sterols, which are not immediately needed, are stored esterified with fatty acids in the form of steryl esters (SE). In the yeast, the esterification reaction is catalyzed by the two acyltransferases Are1p and Are2p. Since SE and other non-polar lipids, especially triacylglycerol (TG), are unable to integrate into phospholipid bilayers, they cluster and form the hydrophobic core of so-called lipid particles/droplets (LP). The biogenesis of LP is still not well studied. Several hypotheses have been discussed, but a budding model from the ER seems to be the most logical. TG and SE are mostly synthesized in the ER. When they accumulate that may form a nascent LP which then buds off the ER. The structure of LP consists of a hydrophobic TG core which is surrounded by SE shells and covered by phospholipid monolayer with some proteins embedded [13]. A minor amount of squalene was also found in LP [14]. However, localization of squalene when it accumulates has never been studied before. Squalene is an unusual lipid; because it is neither a typical storage lipid nor a membrane lipid. It was predicted that squalene might accumulate predominantly in the LP because of its hydrophobic nature. However, localization of squalene in cellular membranes should become a point of interest during this Thesis.

Squalene was considered to be lipotoxic for the yeast. However, there was no direct proof for this property. To address this question we made use of a mutant strain which lacks the four genes *DGA1*, *LRO1*, *ARE1* and *ARE2* encoding the major acyltransferase catalyzing TG and SE formation. The quadruple mutant (QM) mutant was used as a model to study localization of squalene in cells which lack the primary lipid depot, the LP. Since functional gene products of *DGA1*, *LRO1*, *ARE1* and *ARE2* are prerequisites for the biogenesis of LP, a main storage site of non-polar lipids in wild type, the LP, is missing in QM. We hypothesized that squalene if really lipotoxic will either form independent lipid droplets to protect cells from lipotoxicity, or cells will not be viable. As will be shown in this Thesis both predictions were wrong. We observed in this study that squalene was really predominantly located in the LP in wild type cells but also surprisingly accommodated in membranes, especially under conditions of squalene accumulation. Thus, we decided to further elucidate the influence of squalene on the LP structure and on membrane properties in yeast.

The aim of this study was to shed more light on the biochemical and molecular properties squalene. In this Thesis the role of squalene in lipid storage and cell biological consequences of squalene accumulation were addressed. In an introductory chapter of the Thesis, biosynthesis and biochemical properties of squalene in various organisms are

described in the form of a review article. Biotechnological aspects and application of squalene in nutrition and pharmaceutics are addressed as well. In the following two chapters the biochemistry of squalene in the yeast *Saccharomyces cerevisiae* is described with emphasis on storage properties in LP and influence of squalene in membranes. Altogether, this study allows us to evaluate the versatile properties of squalene, an interesting lipid for fundamental research and biotechnology.

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Chapter 1

# Biochemistry and Biotechnology of Squalene

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Key words: squalene, sterol, isoprenoid, mevalonate, methylerythritol phosphate, supercritical extraction, squalene applications

Abbreviations: DMAPP, dimethylallyl diphosphate; DXP, 1-deoxy-D-xylulose-5-phosphate; DXR, DXP reductoisomerase; DXS, DXP synthase; FPP, farnesyl diphosphate; FPS, FPP synthase; G-3-P, Glycerol-3-phosphate; GPP, geranyl diphosphate; HMG-CoA, 3-hydroxy-3-methyl-glutaryl-CoA; HMGS, HMG-CoA synthase; HMGR, HMG-CoA reductase; IDI, isopenthenyl diphosphate isomerase; IPP, isopentenyl diphosphate; MEP, 2-C-methyl-D-erythritol 4-phosphate; MVA, mevalonate; SQ, squalene, SQS, squalene synthase; SQE, squalene epoxidase

## Abstract

Squalene is a natural triterpene and an important intermediate of sterol and hopanoids biosynthesis in various types of cells form bacteria to human. Synthesis and further conversion of squalene are key steps on the metabolism of sterols and related components. These steps are subject to regulation and sensitive to drugs. In this review we summarize the recent knowledge of squalene biochemistry, biotechnology and application of this lipid in nutrition and medicine. Properties of squalene are described and all biological and molecular effects caused by this lipid are discussed.

# Introduction

Squalene (2,6,10,15,19,23-hexamethyl-6,6,10,14,18,20-tetracosahexane) is a triterpenic hydrocarbon. It is widely present in nature and reasonable amounts are found in olive oil, palm oil, wheat-germ oil, amaranth oil and rice bran oil. Its richest source, however, is shark liver oil (60% by weight) which has been traditionally used as source of squalene. In humans, squalene is present at its highest concentration in sebum (~13%).

Squalene is synthesized in all types of cells because it is a key intermediate in the formation of sterols from eukaryotes and hopanoids from bacteria. Squalene and its related compounds, oxidosqualene and bis-oxidosqualene, have been found to be precursors of nearly 200 different triterpene skeletons [1]. Finally, squalene can also be utilized as a carbon source by some microorganisms such as bacteria.

Squalene has several beneficial properties. It is a natural antioxidant [2], serves in skin hydration (for a review see [3]) and has been used as emollient in adjuvants for vaccines [4]. As a compound of olive oil, it has also a preventive effect on breast cancer, posses tumor-protective and cardio-protective properties [5-7], and decreases the serum cholesterol level [8;9]. Moreover, squalenoylation is a common method for delivering prodrugs into cells [10-12].

Due to its high enrichment squalene has been isolated for a long time from shark liver oil. During the last decades this approach has been restricted and isolation of squalene from new sources, such as distillate residues from olive oil, soybean oil, rice bran oil or amaranth oil came under investigation. In these alternative sources squalene is part of unsaponificable by-products of oil refining processes leading to a yield of 3.6% in soybean oil and 30% in olive oil. Isolation of squalene from microorganisms is still under development and investigated on a basic scientific level. As an example for this approach, some squalene accumulating marine-bacteria have been isolated. Initial studies were also performed with the yeast *Saccharomyces cerevisiae*, especially with some mutants accumulating squalene, and with *Pseudozyma sp.*, but the yield of squalene was low. Higher concentrations of squalene were obtained with the microalgae *Traustochytrium sp*. and *Auranthiochytrium sp.* (formerly *Schizochytrium*).

In this review, we will focus on biochemical and biophysical properties of squalene and then address biosynthesis of this compound with emphasis on squalene forming enzymes. We will point out differences of squalene synthesis in different organisms and describe

special properties of the enzymes involved. Finally, we will summarize common protocols of the biotechnological production of squalene and discuss recent applications of this lipid component.

## Biochemical and biophysical properties of squalene

Squalene is a polyunsaturated triterpene, which contains six isoprene units (Figure 1A). At room temperature, squalene is a liquid and has a pleasant, bland taste. In Table 1, some basic properties of squalene such as viscosity, density and solubility are summarized. These data underline the strong hydrophobic nature of this molecule. Due to its chemical structure, especially the high degree of unsaturation, squalene is not very stabile and gets frequently oxidized. In complex mixtures such as olive oil, however, it gains more stability. Nevertheless, Manzi *et al.* [13] observed decomposition of squalene in the range of 26-47% in olive oil after 6 months storage in the dark and at room temperature, whereas other studies described a maximum decrease of 20% even under more severe conditions and during pan-frying [14-18].

In the yeast a small amount of squalene was detected in the highly hydrophobic environment of lipid particles/droplets [19;20] and only in traces in cellular membranes. As can be seen from Fig. 1, double bonds allow squalene to occur in several conformations, e.g., in a symmetric, stretched or coiled form [21]. Most interestingly, squalene can also be organized in the shape of a sterol which may influence its ability to accommodate in a membrane. Hauss *et al.* [22] showed that squalane, a hydrogenated relative of squalene, can be horizontally inserted in a phospholipid bilayer membrane. The authors argued that this conformational arrangement caused protection against proton leakage and affected transmembrane proton flux. Lohner *et al.* [23] demonstrated that squalene at a concentration of 6 mol % in pure phospholipid vesicles altered the lamellar-to-inverse-hexagonal phase transition by increasing the size of inverse hexagonal phase tubes. It was assumed that squalene is rather coiled and stored in the most disordered region of the membrane bilayer. Experiments from our own laboratory (see Chapter 3, Spanova *et al.*, unpublished data) using biological membranes from the yeast and model membranes extended this model insofar as squalene in the ER may rather adapt to a conformation close to ergosterol, whereas in the plasma membrane squalene might be stored in its coiled conformation.

## Squalene in Animal and Human Cells

### *Source and localization*

Squalene is one of the most important lipids of skin cells. There, it is present as a lipid of sebaceous glands where it is synthesized and accounts for 13% of total lipids (for a review see [24]). Its total concentration in the skin [25;26] and the squalene to cholesterol ratio [27] varies with the skin site. Total secretion of squalene was found to depend on the individual varying from 125-475 mg/day [27]. Interestingly, very little of the squalene produced in sebaceous cells is further converted to cholesterol. This effect may be caused either by a low activity of squalene synthase or by down-regulation of oxidosqualene cyclase (squalene epoxidase), which converts squalene to oxidosqualene and is the following enzyme in the cholesterol biosynthetic pathway. Both enzymes of sebaceous glands probably depend on environmental conditions [24].

Squalene in the human organism originates partly from endogenous cholesterol biosynthesis and partly from dietary sources, e.g., olive oil. The intracellular pool of squalene is in equilibrium with the plasma pool [28]. About 60-85% of dietary squalene is absorbed and transported in the serum, mostly together with VLDL, and then distributed to various tissues. Interestingly, a very small amount of squalene (300 mg from 2.4 g) taken up as nutrient is converted to cholesterol. Even higher consumption of squalene does not change the cholesterol level despite of a several-fold increase of squalene in serum [29]. In fact, increased amounts of squalene in serum are safe and beneficial, and showed chemo-preventive and hypocholesterolemic properties [30;31]. A diet containing 850 mg squalene per day for 20 weeks significantly decreased total cholesterol (17%), low density cholesterol (22%) and triacylglycerol (5%) levels in patients suffering from hypercholesterolemia [32].

Squalene at very high concentration can be found in the bodies of cartilaginous fish which lack a swim bladder and must therefore reduce their body density with fats and oils. Squalene is very abundant in sharks (*Squallus spp, Centrophorus squamosus*) and whales (*Physeter macrocephalus*) [33-35]. In shark liver oil, the squalene concentration reaches 40-70% by weight. This extraordinary high concentration resulted in intense sharks hunting process their livers to make health care products. However, due to environmental and marine protection concerns over shark hunting motivated squalene isolation from other sources.

***Biosynthesis of squalene and its regulation in mammalian cells***

Sterols are essential structural and regulatory components of eukaryotic cell membranes. As an intermediate of sterol biosynthesis, squalene plays an important role in that way. Synthesis of squalene is similar in all organisms, although properties of enzymes involved in its formation can differ. In some cases, the reaction leading to squalene formation is catalyzed by single enzyme; in other cases more enzymes (isoenzymes) are involved.

In animal and human cells, cholesterol can be synthesized via *de novo* mevalonate/ isoprenoid pathway or taken up through low density lipoproteins (LDL) which enter the cell via LDL receptors on the cell surface and then get hydrolyzed in lysosomes. There is a clear balance between internal and external cholesterol sources which is governed by feedback control of biosynthetic and uptake pathways. Major players in feedback control mechanisms are 3-hydroxy-3-methyl-glutaryl-CoA reductase (HMGR) and LDL receptors. To prevent cholesterol accumulation, HMGR activity can be reduced by more than 90%, and the number of LDL receptors can be decreases [36], respectively.

*De novo* synthesis of cholesterol (Fig. 2) starts with the mevalonate/isoprenoid pathway to synthesize 3-isopenthenyl-diphosphade (IPP) and farnesyl-pyrophosphate (FPP), which leads then to the formation of squalene and late steps of sterol synthesis. The mevalonate pathway starts with acetyl-CoA, which is converted to 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) and then reducted by HMGR to mevalonate. As mentioned above, the last step of this cascade is rate-limiting and highly regulated. Regulation is maintained by activating or degrading HMGR. HMGR, an integral protein of endoplasmic reticulum (ER) membranes, contains a transmembrane sterol-sensing domain which plays a role in the degradation of the enzyme via proteasome. Recent studies showed direct and indirect stimulation of degradation by cholesterol, lanosterol and oxysterols [37;38]. After phosphorylation and decarboxylation of mevalonate, IPP, the precursor of all polyprenyl compounds, is formed. Subsequently, condensation with another IPP yields farnesyl pyrophosphate (FPP), which can be either converted to squalene and then to sterols or directed towards the synthesis of isoprenylated cellular metabolites, e.g. heme, dolichols and ubiquitin [39-41]. Moreover, FPP is involved in farnesylation of proteins including small GTP-binding proteins like Rho, Ras and Rac [42-44]. Then, squalene synthase combines two FPP molecules to form squalene and thus directs FPP towards cholesterol synthesis [45-47]. The enzyme squalene synthase (SQS) competes with other enzymes for the FPP substrate and was shown to respond to the cellular sterol content in a similar manner as HMGR (reviewed in [47]). SQS regulates synthesis of other essential non-sterol isoprenoids by redirecting FPP

to their pathways [48;49]. In the following enzymatic step, epoxidation of squalene to squalene 2,3-oxidosqualene catalyzed by squalene epoxidase (squalene monooxygenase, SQE) occurs [50;51]). For the activity of this enzyme, a cytosolic (S105) fraction, molecular oxygen, NADPH-cytochrome c reductase, NADPH and FAD are needed [52-55]. This enzymatic step has been well studied, because inhibitors (statins) of this reaction exhibit beneficial effects such as lowering LDL cholesterol (reviewed by [56]). SQE is affected through a feedback mechanism by sterols, but not by non-sterol intermediates of the mevalonate pathway [57]. 2,3-Oxidosqualene is then further converted to cholesterol in several steps which will not be discussed here in more detail.

## Squalene in plants

### *Source and localization*

Besides shark liver oil several plants such as olives became valuable sources for the biotechnological isolation of squalene. Smaller amounts of squalene are also present in palm oil, wheat germ oil, peanut oil, amaranth oil, rice brain oil, fish oil and animal fat [5;58].

Olive oil is the main source of squalene with a yield of 7 mg squalene per g oil [5]. Together with oleic acid (72%) and polyphenols squalene has been found to be beneficial, although olive oil does not contain any omega-3 or omega-6 polyunsaturated fatty acids. The mixture of lipid components in olive oil appears to contribute significantly to health with more effects on prevention than treatment (reviewed [5;59;60]). Decreased risk of breast, skin and colon cancer [60;61], a chemo- and cardio-protective effect [5;6;60], an antihypertensive effect [62;63], anti-inflammatory action [64] and prevention of atherosclerotic plaque formation [65] were reported. The protection, however, depends on the amount and time period of olive oil consumption. Interestingly, annual olive oil consumption per person can be up to 15 kg [60].

### *Biosynthesis of squalene in plants and its regulation*

In plants, the biosynthetic pathway of sterols is slightly different from animal cells and fungi. Biosynthetic reactions from squalene to phytosterols result in formation of various sterols such as sitosterol, stigmasterol, campesterol and isofucosterol. In plants, squalene is oxidized to 2,3-oxidosqualene and then converted to cycloartenol, which is further metabolized to the end product of this biosynthetic cascade, sitosterol (reviewed in [66-68]).

Phytosterols synthesized in the endoplasmic reticulum are rapidly transported mostly to the plasma membrane and to a minor extent to the Golgi [69-71].

Isoprenoids can be synthesized via mevalonate pathway in the cytosol (e.g. sterols and brassinosteroids) and mitochondria (e.g. side chains of ubiquinone), or via 2C-methyl-D-erythritol-4-phosphate (MEP) pathway (formerly known as non-mevalonate pathway, DXP pathway [72]) in plastids (e.g. carotenoids and the side chains of chlorophylls, plastoquinones and isoprenoid-type phytohormones) [72;73] (Figure 3). Whereas the MVA pathway forms only isopentenyl diphosphate (IPP), the MEP pathway generates IPP and dimethylallyl diphosphate (DMAPP). Exchange of isoprenoids between the cytosol and plastids is marginal (reviewed in [68]), probably due to the posttranscriptional processes [74].

Mevalonate pathway and squalene synthesis in plants are similar to vertebrates and fungi (Figure 3) with three important steps catalyzed by HMGR, farnesyl pyrophosphate synthase (FPS) and squalene synthase (SQS). The number of genes encoding HMGR varies from two described for *Arabidopsis thaliana* [75;76] to at least eleven in potato [77;78]. HMGR was found to be the crosstalk enzyme for sphingolipid and sterol biosynthesis [79]. An *Arabidopsis hmg1* mutation exhibited dwarfism, early senescence and male sterility; *hmg2* had no visible phenotype [80], but complete deletion was lethal for male gametophytes [81]. As in mammalian cells, HMGR is controlled through feedback regulation in response to selective depletion of endogenous sterols [82]. In tobacco cells, up-regulation of HMGR led to overproduction of sterols [83], which were stored together with fatty acids in the form of steryl esters in lipid droplets, called sterolosomes. Overexpression of other enzymes of the pathway, e.g., FPS1S [84], did not exhibit any or only a minor effect on the total amount of sterols.

The MEP pathway starts with condensation of glyceraldehyde-3-phosphate and pyruvate to form 1-deoxy-D-xylulose 5-phosphate (DXP) catalyzed by DXP synthase (DXS, formerly CLA1). DXP serves as a precursor in the thiamine and pyridoxol biosynthesis in bacteria and plastids. DXS is the feedback regulation point of this pathway [85;86], and its deletion resulted in seedling-lethal albino phenotype which was rescued by addition of DX, declaring the MEP pathway to be essential for plants [87-89]. Two additional genes with homology, DXS-like (DXL), were found in green siliques (*DXL1*, formerly *DXS2*) and roots (*DXL2*, formerly *DXS3*) to encode DXS. Since *cla1*/*dxs-1* deletion was not rescued and showed the above mentioned albino phenotype, probably none of them is redundant to *DXS*. The rest of the MEP pathway steps seem to be encoded by single genes, each. MEP is formed via reductive isomerization catalyzed by 1-deoxy-D-xylulose 5-phosphate reductoisomerase

(DXR). MEP is further converted to 4-(cytidine 5′-diphospho)-2-*C*-methyl-d-erythritol in CTP-dependent mechanism by the *MCT* gene product 2-*C*-methyl-d-erythritol 4-phosphate cytidylyltransferase (MCT). Phosphorylation by 4-(cytidine 5′-diphospho)-2-*C*-methyl-D-erythritol kinase (CMK) and following cyclization after loss of the cytidyl group yields 2-*C*-methyl-d-erythritol 2,4-cyclodiphosphate in a reaction catalyzed by 2-*C*-methyl-d-erythritol 2,4-cyclodiphosphate synthase (MDS). Two last steps are catalyzed by (*E*)-4-hydroxy-3-methylbut-2-enyl diphosphate synthase (HDS), and reductase (HDR) and then IPP and DMAPP are formed. The expression of *DXS, DXR, MCT, CMK, MDS, HDS* genes is induced by light, whereas the expression of the *HDR* gene is constitutive in *Arabidopsis* seedlings [90]. It is noteworthy that all null mutants of genes *DXR, MCT, HDS* and *HDR* [87;90-93] exhibited the albino phenotype which suggested that chloroplast development is arrested at early stages in these mutants.

Condensation of two molecules of IPP with dimethylallyl diphosphate producing FPP is catalyzed by farnesyl pyrophosphate synthase (FPS). In plants, FPP serves as substrate for the synthesis of phytosterols, dolichols, ubiquinons, heme *a*, sesquiterpenoid, phytoalexins or abscisic acid. *Arabidopsis thaliana* contains three FPP isoenzymes, mitochondrial FPS1L, cytosolic FPS1S and FPS2 [94;95]. FPS1L and FPS1S differ only at the N-terminus. FPS1S and FPS2 have different patterns of expression. While FPS1S is expressed in most plant organs and during most of the plant cycle, FPS2 is expressed strongly during seed development [94;96]. Single FPS mutations did not show any major effect because isoenzymes compensated the defect. Lack of FPS2 isoenzyme causes HMGR upregulation in seeds which compensates for the low expression of FPS1 during seed development. The *fps1fps2* double mutant was viable, but resulted in arrested embryo development at the pre-globular stage [96]. Two molecules of FPP condense and form squalene via presqualene diphosphate catalyzed by squalene synthase (*SQS*) [97]. In *Arabidopsis thaliana, SQS1* and *SQS2* encode for two squalene synthases, but only the gene product of *SQS1* shows enzymatic activity. *SQS1* is expressed in all plant tissues and targeted to the endoplasmic reticulum membrane [98]. It can be specifically inhibited by squalestatin, also called zaragozic acid [99]. Squalestatin has been used to investigate the isoprenoid pathway in plants because it represents a good tool to redirect FPP towards the non-sterol isoprenoid pathway.

Squalene epoxidase (*SQE*) catalyses conversion of squalene to 2,3-oxidosqualene. In contrast to mammals and yeast, plants have multiple genes predicted to encode *SQE* suggesting specific regulation. One of six putative *Arabidopsis SQE* genes, *SQE1*, is essential for normal plant development and regulates root and hypocotyl elongation [100;101]. It is

involved in drought tolerance and regulation of the amount of reactive oxygen species [101]. Mutants deleted of *SQE1* accumulate squalene, have elongation defects and are not able to create viable seeds. The gene product of *SQE2* produces primary 2,3-oxidosqualene, whereas those of *SQE1* and *SQE3* also can synthesize 2,3:22,23-dioxidosqualene. Triterpenoid synthesis may also be associated with mitochondria, since *SQE2* and one putative squalene epoxidase from rice have predicted mitochondrial targeting sequences. Moreover, *Arabidopsis FPS1* is a mitochondrial protein [95;100]. *SQE4*, *SQE5* and *SQE6* have specific although hypothetic functions, e.g., in plant defensive mechanism of rice [102;103]. Inhibition of SQE with terbinafine leads to accumulation of squalene which is stored mostly in lipid particles from where it can be mobilized when needed [82].

## Squalene in microorganisms

Microbial squalene production has become a promising alternative to other sources mentioned above. Although microorganisms do not accumulate as much squalene as plants or shark liver, their advantage is fast and massive growth. Squalene isolation from yeast [104], especially *Saccharomyces* [105-108], *Torulaspora delbrueckii* [109], *Pseudomonas*[110], *Candida* [111], the algae *Euglena* [112] and the microalgae *Traustochytrium* [109;113], *Schizochytrium mangrovei* [114;115], and *Botryococcus braunii* [116] was reported.

### *Squalene synthesis in prokaryotes*

The synthesis of squalene differs in bacteria depending on species (listed in e.g. [117]). Squalene precursors isopentenyl diphosphate (IPP) and dimethylallyl diphosphate (DMAPP) are synthesized either via MVA, MEP or both pathways by different mechanism (for detailed information see [118]). The MEP pathway occurs mostly in all eubacteria, cyanobacteria and apicomplexan parasites such as *Plasmodium* whereas MVA was found in archaea, a few eubacteria, fungi and *Trypanosoma* and *Leischmania* [40]. Obligate parasitic eubacteria such as *Rickettsia* or *Mycoplasma* do not use any of these mechanisms and obtain their isoprenoids most likely from host cells [119;120].

The eubacterial MEP pathway is similar to plants. Enzymes of the MEP pathway have been identified and characterized (reviewed [73;91;117;118;121;122]). DXS is a well studied enzyme which plays a limiting role in the isoprenoid pathway [123-127]. Instead of G-3-P and

pyruvate it can utilize another substrates, sugar phosphates, short aldehydes or α-ketoacids hydroxypyruvate and α-oxobutyrate [128]. Recently, new family of DXR which catalyzes the conversion of DXP to MEP was reported in bacterium *Brucella abortus* [129]. Some of the bacteria lack DXR, but they have enzyme DRL (DXR-like) to catalyze the same reaction. Some bacteria posses both enzymes [129].

Ershov *et al.* [130] observed that inhibition of the enzyme DXR from the MEP pathway in cyanobacteria did not affect isoprenoid biosynthesis under photosynthetic conditions. They proposed alternative substrate from pentose phosphate cycle derived from photosynthesis which might enter the isoprenoid MEP pathway downstream of MEP. Moreover, following inactivation of IDI type II provided further evidence for more complex isoprenoid biosynthesis, where even DMAPP could be synthesized through a separate pathway [131]. This finding is in contrast with the typical MEP pathway found in *E. coli*. In contrast to plants, many bacteria posses two types of isopentenyl diphosphate isomerase, IDI-type I and type II [132-134]. Type I depends on divalent cations whereas IDI-II requires metal ion, FMN and (under anaerobiosis) NADPH.

Most archaebacteria use the MVA pathway for the synthesis their membrane ether-linked isoprenoid lipids (*Halobacterium cutirubrum, Caldariella acidophilus* [135;136]), but using only three enzymes of MVA, *hmgs* (HMG synthase), *hmgr* (HMGR), *mk* (mevalonate kinase). Moreover, IDI type II was detected in *archaea*.

Most *Streptomyces* species use the MEP pathway for the biosynthesis of IPP [137]. In some *Streptomyces* species, the complete MEP and MVA pathway was found. It was shown that the MEP pathway was used for primary metabolic function whereas the MVA pathway played a non-essential role synthesizing secondary metabolites [138;139]. Based on these observations and results of lateral gene transfer it seems that the MEP pathway is older than the MVA pathway [122].

IPP and DMAPP formed as described above are further condensed to FPP. Conversion of FPP to squalene is catalyzed by SQS. The sequence of SQS from *Thermosynechococcus elongatus* BP-1 has only approximately 30% similarity with eukaryotic SQS, but the isolated protein showed the same pH dependence, metal ion dependence and kinetic properties as eukaryotic SQS and was also inhibited by zaragozid acid [140].

In prokaryotes, the synthesis of components after squalene varies. In bacteria, different class of triterpenoids, pentacyclic hopanoids, are formed as building blocks for membrane biogenesis [141;142]. Hopanoids are most abundant in aerobic bacteria (cyanobacteria, methanotrophs and heterotrophs) and in some anaerobic bacteria, but not in archaea.

Hopanoids play a role in maintaining membrane integrity and permeability [143] and cope with external stress such as ethanol tolerance [144], oxygen diffusion [145], and prevention of water diffusion into spores [146]. The synthesis of hopanoids starts from squalene and is catalyzed by squalene-hopene cyclase. The existence of this reaction, however, does not exclude synthesis of steroids by S-2,3-oxidosqualene cyclase. Interestingly, co-existence of both enzyme activities has been demonstrated in *Methylococcus capsulatus* [147]. Bode *et al.* [148] screened for squalene and steroid production in myxobacteria. They concluded that steroid patterns are species and strain specific and not affected by inhibitors of the steroid biosynthetic pathway in eukaryotes. Recently, Lamb *et al.* [149] showed the presence of a postsqualene lanosterol biosynthetic enzyme complex acting in the prokaryotic sterol pathway. Thus, some bacteria appear to be able to synthesize sterols. These data might be a key to understand phylogenetic route of sterol and steroid synthesis.

Interestingly, squalene can be used as a carbon source by some bacteria such as *Corynebacterium sp.* [150;151], *Corynebacterium terpenotabidum sp. nov.* [152], *Rhodococcus sp.* [153], *Pseudomonas sp.* [154] or *Arthrobacter sp.* [155;156]. Mechanisms involved in this process appear to include (i) oxidation of the terminal methyl groups and formation of the corresponding α,ω-diodic acid [151], (ii) hydration of the double bonds resulting in tertiary alcohols [150], or (iii) cleavage of internal double bonds catalyzed an oxygenase leading to geranylacetone and 5,9,13-trimethyltetradec-4E,8E,12-trienoic acid [155;156]. *Marinobacter sp.* (2sq31) is able to degrade squalene under aerobic and anaerobic conditions [157]. The proposed model is that denitryfing bacteria under anaerobiosis hydrate first squalene to methyl ketones and alcohols, which are carboxylated to isoprenoid acids and then metabolized via β-oxidation and β-decarboxymethylation [154;158-160]. Aerobic degradation starts with cleavage of C10/C11 or C14/C15 double bonds in addition to steps of the anaerobic pathway as observed with *Arthrobacter sp.* [155].

Recently, studies about improving squalene production in *E. coli* were published. Ghimire *et al.* [161] introduced and overexpressed putative hopanoid genes from Streptomyces peucetius ATCC 27952 *hopA*, *hopB* (coding for squalene/phytoenol synthase) and *hopD* (encoding farnesyl diphosphate synthase). The yield of squalene was increased from 4.1mg/L to 11.8 mg/L with additional introduction and expression of genes of deoxyxylulose phosphate synthase and IPP isomerase.

***Squalene synthesis in yeast***

Much work in defining the role of sterols in eukaryotic cells has been done using the yeast as a model system. Effects of sterols on membrane fluidity [162], membrane permeability [163;164], energy source utilization [165] and the activity of membrane-bound ATPase [166] were demonstrated using yeast mutants bearing defects in the ergosterol biosynthetic pathway. As in other eukaryotic cells, the formation of sterols in yeast can be divided into two parts. The first part named mevalonate or isoprenoid pathway starts with acetyl-CoA and ends with farnesyl pyrophosphate (FPP), which is used as substrate for further biosynthetic routes, e.g., synthesis of heme [167], quinones [168] and dolichols [169]. Mutations affecting these steps are lethal.

The best studied enzyme of the mevalonate pathway is HMGR, the first control point of regulation. Actually, yeast cells harbor two HMGR enzymes encoded by *HMG1* and *HMG2*, respectively [170]. It has been shown that overexpression of *HMG1* leads to an approximately 40-fold increase in enzymatic activity of HMG-CoA reductase, a higher yield of the dry matter and accumulation of squalene [171]. The enzyme shows feedback inhibition similar to animal and plant cells in the presence of ergosterol [172] and is subject to catabolic repression [173]. Although Hmg1p and Hmg2p are functionally similar, regulation of gene expression was shown to be different for the two isoenzymes. Thorsness *et al.* [174] found that expression of *HMG1* was stimulated by heme, whereas expression of *HMG2* was repressed by heme. Deleting *HMG1* a *HMG2* rendered yeast cells non-viable because they could not form mevalonate [170].

The conversion of FPP to the end-product ergosterol includes eleven reactions. Mutations in the first three steps are lethal and there are a few well characterized points of regulation in this pathway. Fusion of two FPP molecules yielding one molecule of squalene is catalyzed by the squalene synthase Erg9p [175]. Similar to HMGR, Erg9p is subject to transcriptional regulation [176]. In the yeast, squalene does not accumulate within the cell under regular conditions because squalene is efficiently converted to ergosterol. A minor amount of squalene was detected in lipid particles forming the depot of non-polar lipids together with triacylglycerols and steryl esters [20]. When squalene accumulates under anaerobiosis or in *hem1* mutant cells, over 70% of its cellular amount is accumulated in lipid particles. Small amounts of squalene were found in membranes [19;177]. It was shown that squalene accumulation did not cause a lipotoxic effect[19].

Several biosynthetic pathways, such as the synthesis of heme, sterols, unsaturated fatty acids, pyrimidines and deoxyribonucleotides [178-181] require molecular oxygen. Therefore,

ergosterol and unsaturated fatty acids are required as supplement for viability of yeast cell grown anaerobically [180;181]. Under these conditions squalene accumulates at a maximum yield of 41.16 mg/kg dry weight (DW) when cultured at 25°C [109;113]. Recently, various conditions and the inoculum size were tested to increase the yield and productivity of squalene [182]. It has to be taken into account, however, that strictly anaerobic cultivation for yeast squalene production is difficult. Jahnke and Klein [183] observed that squalene epoxidase (Erg1p) activity increased to almost half of its maximal value in anaerobic yeast after adding 0.03% oxygen and rapid synthesis of ergosterol from squalene occurred. This problem may be overcome by additional genetic mutations, e.g., deletions of *ERG1* or *ERG7*.

Squalene of the yeast also accumulates under heme-deficiency [19]. Blocking the synthesis of heme leads to accumulation of lanosterol since sterol-14-α-demethylase (Erg11p) is the first NADPH-heme-dependent cytochrome P450 protein in the biosynthetic sequence of ergosterol [184]. At the same time, however, squalene accumulates at substantial amounts. It has to be noted that only under anaerobiosis, in heme-deficient strain and sterol uptake mutants, e.g. upc2-1 [185;186] ergosterol can be properly taken up by the yeast.

Under aerobic conditions, squalene accumulation can also be achieved by increasing the rate of flow through the early part of the mevalonate pathway, which can be mostly accomplished by increasing the specific activity of HMGR [171;187]. Loertscher *et al.* [188] showed that the sterol composition of a mutant overexpressing *HMG1* mutant changed with increasing temperature. Cells grown at 16°C stored higher amounts of squalene (approximately 4-fold) compared to cells grown at 30°C. Thus, temperature shift is another possibility how yeast cells can accumulate squalene.

In the ergosterol biosynthetic pathway, squalene formed through reactions described above is further converted to squalene epoxide by the squalene epoxidase Erg1p [189]). This step requires oxygen making ergosterol synthesis strictly aerobic [183]. Erg1p is dually localized in yeast, namely in the ER and in LP [190]. *In vitro*, enzymatic activity of squalene epoxidase was only detectable in the ER, but was absent from isolated LP [190]. Probably, a reductase required for this reaction might be localized to the ER, but missing in LP. The subsequent step of ergosterol synthesis is cyclization of 2,3-oxidosqualene and formation of the first sterol, lanosterol (Fig.3). Lanosterol synthase (oxidosqualene cyclase), which is encoded by the *ERG7* gene, is catalyzing this reaction [191;192]. The remaining steps of the ergosterol biosynthetic pathway include modifications of the sterol ring system and of the side chain. We will not describe details here, and the reader is referred to review articles addressing these aspects [193-195].

***Squalene synthesis in microalgae***

Another cellular alternative to produce squalene are microalgae which are microscopic algae, typically found in fresh water and marine systems [196]. They were widely used for production of various compounds such as polysaccharides, proteins, carotenoids, enzymes etc, or as a source for renewable energy transforming sewage and waste into valuable biomass.

Strains of *Botryococcus braunii* are divided into 3 classes A, B and L depending on types of synthesized hydrocarbons. B types synthesize triterpenoids such as methylated squalene and hydrocarbons botryococcenes. In contrast to fungi, IPP of microalgae is synthesized via MEP pathway [197;198]. FPP has been suggested as a precursor of botryococcenes [199]. Squalene synthase has been identified, but it is still unclear whether this enzyme catalyzes squalene or botryococcene synthesis, or whether are two separated enzymes [199;200].

Some microalga can accumulate reasonable amounts of squalene [114]. Traustochytrid *Aurantiochytrium sp*. (formerly known as *Schizochytrium*) is an efficient producer of squalene, it excel in rapid growth and high squalene content under heterotrophic conditions, which eliminated the usual problem of microalgae with light limitation in closed culture systems [201;202]. Moreover optimizing of culture ingredients led to increase of its content and yield [203].

In microalgae, accumulation of squalene can be achieved by varying culture conditions. Jiang *et al.* [114] reported that the squalene level depends on the cultivation time. While the squalene level reached 0.162 mg/g DW after 3 days, only 0.035 mg/g DW was found after 5 days in cultures of *Schizochytrium mangrovei*. These results of cultivation time dependency were in line with reports about squalene production in *Saccharomyces cerevisiae*, *Torulaspora* sp. and *Traustochytrium* sp. [109;204]. *Botryococcus braunii* was also found to accumulate squalene, but this microorganism did not grow well under heterotrophic conditions making this system less attractive for biotechnological production [116]. Chen *et al.* [203] optimized the nitrogen source for enhanced squalene production in *Aurantiochytrium sp.*. They showed that monosodium glutamate, yeast extract and tryptone can enhance squalene synthesis up to 26.3% in content and 10.1% in yield compared to the original medium [201;203].

## Biotechnological aspects of squalene production

Production of squalene is still carried out preferentially from plant or animal sources as starting material. Due to environmental concerns squalene production from shark liver oil has recently been challenged, and other sources such as plants and microorganisms became more important. Extraction of squalene from olive oil deodorization distillate (OODD) became very popular since squalene concentrations in this source reach 10-30%. Alternatively, amaranth grains are used where the squalene content reaches 8% [205-207] or leaves of the tree *Terminalia catappa* [208] or lotus (*Nelumbo nucifera* Gaertn) bee pollen [209;210]. Attempts of large scale production of squalene from microorganism and algae are still in their infancy.

Squalene is thermolabile because of its unsaturated linear chain. Thus, distillation is not a suitable separation process, e.g. from vegetable oils. Moreover, thermal degradation of other compounds of oils such as triacylglycerols may occur as well. There are, however, several alternative ways to extract squalene from natural sources, e.g., solvent extractions or supercritical fluid extraction. The former method, solvent extraction, is very efficient. Squalene as a non-polar lipid can be extracted most efficiently using organic solvents such as hexane. However, this method is largely restricted to research laboratories due to regulatory, financial (expensive) and safety concerns (toxicity and flammability). The method used more frequently in industry is short-path distillation, a high vacuum process. Condensers are positioned close to the evaporator surface and the feed liquid flows on the evaporator surface as a thin falling film. Different temperatures and vacuum settings can be use to distill the determined compound [211]. The third extraction method, supercritical fluid extraction (SFE), is preferred in industry. As the usual solvent, supercritical carbon dioxide (SC-$CO_2$) is used. $CO_2$ gets fluid when it reaches a temperature of 31.1°C and a pressure of 72.9 atm. Due to the near-ambient critical temperature of $CO_2$, SC-$CO_2$ is suitable for extraction of thermolabile natural products. This extraction showed several advantages such as high purity of the product and the potential to extract and concentrate in one step. This process is not very expensive and yields squalene at natural quality without usage of solvents. The efficiency of the extraction can be improved by changing conditions. Co-solvents, e.g. 10-15% ethanol can be used to increase the yield. As some disadvantages of this method, however, complexity of equipment and maintenance of pressure (~10MPa) and temperatures (40-90°C) need to be mentioned. Finally, cells need to be dried to get higher yield of the extracted compound.

As mentioned above, squalene is mostly produced from its most abundant source, the shark liver oil [212]. The extraction of squalene from shark liver oil is limited because of the environmental pollutants (polychlorinated biphenyls, dioxins and heavy metals) presented in shark liver [213;214]. Moreover, shark liver oil contains 0.1% pristane, low volatile triacylglycerols and glyceryl ethers. Removal of pristane ($C_{19}H_{40}$) is needed since it is a skin irritant. Under optimum processing conditions squalene was obtained at 95% purity by weight without using reflux and at 99% purity with reflux. Recent investigations using short-path distillation led to odor-free 97% pure squalene [33].

Olive oil deodorization distillate residues (OODD) are by-products of the oil refining process and contain 10-30% squalene and 30% free fatty acids (FFA) by weight besides some sterols and tocopherols. They are usually mixed with olive oil neutralization by-products and hence have a low market value. A typical flow-sheet of counter-current packed column extraction used for squalene extraction is shown in Figure 4. The technical problem of counter-current packed column extraction with SC-$CO_2$ is separation of FFA and squalene because of very similar solubility in SC-$CO_2$. Thus, additional steps are needed for the purification of squalene. Ruivo *et al.* [215] tried to overcome this problem with model mixtures of squalene and oleic acid by introducing nanofiltration with six different membranes taking into account the different molecular weights of squalene and oleic acid. The idea was that FFA as smaller molecules with higher diffusivity in SC-$CO_2$ would permeate membranes and squalene would concentrate in the retentate. Surprisingly, squalene permeated membranes preferentially over oleic acid. This effect was explained by the specific interaction of permeating molecules and the active layer of the membrane. The highest selectivity was obtained using polydimethyl siloxane and polyamide AD membrane, but the first one showed the lowest efflux (30%) [215]. Another strategy was used by Bondioly *et al.* [216], who separated squalene from glyceride and non-glyceride substances normally found in olive oil. FFA, fatty acid methyl and ethyl esters were converted to their corresponding triglycerides prior to SFE and then easily separated, as suggested for TG/oleic acid mixture [217]. This separation process requiring zinc catalytic esterification and high pressure yields highly enriched squalene.

An alternative to counter-current packed column separation are static mixers. As an example, static mixers are used for the removal of caffeine from SC-$CO_2$ by water after SFE decaffeination [218]. Lower costs, short residence times and minimal space requirements compared to the packed column led Catchpole *et al.* [219] to focus on supercritical extraction of lipids in a static mixer on laboratory and pilot-scale. Although the separation factor

between squalene and other major components did not reach the number achieved in packed column for mixtures easy to fractionate such as shark liver oil, the separation factor in difficult fractionated mixtures (e.g. OODD) showed similar separation in static mixer and in packed column.

Soybean oil deodorizer distillate residues do not contain much squalene but can still be used as a reasonable source. This material usually consists of 3.5% squalene, 13-14% tocopherols (vitamin E), 26% sterols, FFA, triacylglycerols, diacylglycerols and monoacylglycerols [220]. Wang *et al.* [221] concentrated squalene from 80% in a feed mixture up to 98% in the product in an additional cleaning step, the adsorption step by pressure swing adsorption in SC-$CO_2$.

The deodorization distillate of rice bran oil contains 8% squalene as another possible source. Many patents of fractionating squalene or squalane from such deodorization distillates have been published [222-224]. Isolations methods include saponification, solvent fractionation, distillation, hydrogenation and finally molecular distillation. Sugihara *et al.* [225]) reported recently a new fractionation method of squalene and phytosterols which is based on a combination of solvent fractionation and supercritical fluid chromatography (SFC) after SFE of the deodorization distillate. This method brought many advantages, e.g. fewer operation processes, time-saving, no oxidative rancidity and continuous production of the two functional components.

Another plant source rich in squalene is amaranth seed oil (*Amaranthus cruneus*). Oil from amaranth grains containing 6-8% of squalene [205-207] was used for concentrating this compound. As an isolation method short-path distillation was employed (180°C, 3mtorr vacuum) resulting in 76% recovery of squalene in the distillate [226]. Squalene was also found in leaves and but not in seeds of *Terminalia catappa*, a tropical and sub-tropical tree used in folk medicine for its antipyretic and hemostatic properties and prevention of hepatitis and hepatoma [227;228]. The squalene content in leaves increases with the increase of maturity. Using SFE a squalene yield of 12.2 mg/g and a content in extracts of 12.29 % were obtained [208].

Production of squalene from microbial sources is still under investigation. As mentioned above, microorganisms have a great potential to become major sources for squalene isolation. Advantages as rapid and massive growth, however, still do not compensate for the low squalene yield. Current investigations address the increase of the squalene in microorganism. *Saccharomyces cerevisiae* as one of the best studied eukaryotic organisms is still a not good enough to produce squalene at large scale. Genetic mutations may be a

strategy for an improvement. Other microorganisms showed a more promising accumulation of squalene. *Torulaspora delbrueckii* accumulated 237.25 mg squalene/kg DW [109]; and *Pseudozyma sp.* produced up to 5.20 g/L of biomass and 340.52 mg/L of squalene [229]. Some marine-bacteria were found to accumulate squalene, such as *Rubritalea squalenifaciens* sp. nov. [230], *R. sabuli* sp.nov. [231], *R. spongiae* [232] and *R. tangerine* [232], but were not used for biotechnological processes.

## Applications of squalene

Applications of squalene have been recently reviewed in some detail [5;30;233]. In the following section, we will address some of these applications focusing on the use of squalene for therapeutic, pharmacological and cosmetic purposes.

***Antioxidant***

Previous studies had shown that squalene is can act as a highly efficient singlet oxygen scavenging agent [2;234]. Therefore, squalene was considered to posses antioxidant properties. Since oxidative stress and increase in reactive oxygen species (ROS) may induce cancer [235], squalene was also regarded as a potential anti-cancer component [5]. However, the scavenging capacity of squalene has not been studied in much detail. Warleta *et al.* [236] did not observe antioxidant activity of squalene against 2,2-diphenyl-1-picrylhydrazil stable radicals, 2,2'-azino-bis(3-ethylbenzthiazoline-6-sulphonic acid) cation radicals or 2,2'-azobis (2-methylpropinamid) dihydrochloride-induced peroxyl radicals, even at high concentration of squalene. Similar observation were published before [16;237] concluding that squalene antioxidant activity is extremely low. Conforti *et al.* [238] described an antioxidant effect of squalene with an $IC_{50}$ value of 0.023 mg/ml. Squalene reduced the rate of oxidation in a crocin bleaching assay where it might act as a competitive compound to tocopherol and sitosterol also studied [239]. A weak antioxidant activity of squalene was also observed in olive oil [237], which may, however, be due to the competitive oxidation of the various other lipids present in these samples. Dessi *et al.* [113] studied the effect of squalene on the oxidative stability of PUFA and reported squalene having antioxidant properties as a peroxyl radical scavenger in mild UVA-mediated PUFA oxidation. Combination of PUFA and squalene led to a decrease level of lipid peroxidation in heart tissue of rats [240].

Interestingly, different antioxidant effects of squalene in different types of cells were observed. Squalene showed antioxidant activity *in vitro* only in mammary epithelial and bone marrow cells, but not in human breast cancer cells and neuroblastoma cells [236;241], although reference antioxidants showed antioxidant activity in all tested cells. Warleta *et al.* [236] concluded that the squalene antioxidant selectivity depends on either (i) the "glutathione paradox", where squalene increases the amount of glutathione in normal cells [242]; or (ii) differences in squalene uptake, utilization and accumulation [241]; or (iii) deregulation of antioxidant systems in tumor cells [243]. These data led to the conclusion that squalene acts more on the prevention than on direct treatment of cancer.

***Squalene as a dietary supplement has a preventive effect***

Hyperlipidaemia is considered as a high risk factor of obesity and hypertension [244-246]. Squalene was suggested to be a possible component for the treatment of cardiovascular diseases. It has been shown that squalene reduces cholesterol and triacylglycerol blood level and thus decreases plasma leptin [247]. Moreover, high dosage squalene treatment decreased body fat and glucose in dogs and rats [248-250]. Mechanisms of the squalene effect are still not clear. It could either be a direct effect or indirect through lowering triacylglycerols and thus enhancing body sensitivity to leptin [247;250]. Squalene was also shown to increase testicular weights and testosterone levels in dogs and rats [248-250], improved the reproductive performance of meat-type male chicken and increased the serum testosterone level and semen collection volume. Squalene did not decrease the egg fertile rate in an artificial insemination model but increased the egg fertile rate in a natural mating model [251]. Finally, Motawi *et al*. [252] studied the role of squalene on oxidative cardiac, urotoxic and testicular damage induced by cyclophosphamid in male Wistar rats. These authors found that squalene treatment had a cytoprotective effect and attenuated cyclophosphamid-induced pathological alterations.

***Squalene as a reagent in human medical treatment***

High dose of squalene (>13.5g/day) significantly decreased wrinkles in photo aged human skin, increased type I procollagen and decreased UV-induced DNA damage in human skin *in vivo* but was associated with transient adverse effects such as loose stool [253]. Squalene exhibited antitumor activity against colon, skin, sarcoma and lung cancer in rodents [6;31;254;255]. As an example, olive oil consumption decreased incidence of breast cancer

[60;256], but squalene did not induce death of breast tumor cells and thus may be ineffective once breast tumor has established [236].

The mechanism proposed for the effect of squalene is inhibition of HMGCoA reductase catalytic activity. This mechanism affects Ras p21 farnesylation, signal transduction and cellular proliferation [5]. A novel facet of squalene antitumor activity has been suggested by Newmark and collaborators [5] based on results presented by Strandberg *et al.* [257]. These authors showed that feeding of squalene resulted in a 20-fold increase in the serum and a 30-fold total increase in of methyl sterols, including lanosterol, 14-desmethyl lanosterol and 14-monomethylated sterols. Katdare *et al.* [258] tested lanosterol and other metabolites of squalene as potential antitumor inhibitors. They concluded that squalene metabolites or unphosphorylated precursor substances for posttranslational modifications of Ras p21 oncogenes showed stronger chemopreventive effect than squalene itself.

Squalene has also been frequently used as an additive for lipid emulsions as drug carrier in pharmaceutical and vaccine applications (recently reviewed [4]). Such emulsions are able to incorporate poorly soluble drugs within their dispersal phase, which is beneficial in increased drug and vaccine uptake into cells, minimizing side effects through contact of drug and body fluid and decreasing the release of the drug and multiple adjuvant effects [4]. Squalene and squalane form very stabile and viscose emulsions to solubilize lipophilic drugs, adjuvants and vaccines with most potent transfection activity [259;260] and small droplet size [261] (for reviews see [3;4;233]). Some of these emulsions have been well studied, such as SAF, MF59®, DETOX® and PROVAX® whose composition of vaccine adjuvants were reviewed in [4]. As an example, squalene together with detergents Tween® 80 and Span® 85 is an ingredient of the adjuvant MF59® (Novartis) which is currently the only oil-in-water microemulsion approved for human use [262;263]. MF59® has been shown to be a potent and safe adjuvant with several vaccines, e.g. against hepatitis B and C, herpes simplex virus, HIV-1 and influenza (vaccine Fluad®).

Utilization of squalene for vaccination was not without problems (reviewed [264]). After the Gulf war, veterans showed multiply syndromes, such as rashes, headaches, asthralgias, memory loss, increased allergies, sensitivities and neurological abnormalities [265]. Squalene was suggested to be a cause of this Gulf war symptoms since it was added as adjuvant to the anthrax vaccine [266]. Anti-squalene antibodies (ASA) were found in gulf war syndrome-like patients [267]. Later, it was concluded that ASA occur naturally in humans and are not correlated with anthrax vaccine [268]. MF59 emulsion adjuvant in vaccines did neither induce the level of ASA nor enhanced pre-existing ASA titres [269].

Recently, a prodrug strategy for improved delivery of nucleotide analogs became a research focus. Nucleotide analogues acting is a potent inhibitors of DNA synthesis have been used as antiviral and anticancer therapeutics [270;271]. Transport of these drugs into the cell is highly limited due to high hydrophobicity or poor *in vivo* stability. Squalenoylation of the nucleotide analogues can help to overcome slow diffusion. Conjugation of squalene to the drugs created nanoassembly without using surfactants [272]. As an example, interaction of the lipophilic prodrug gemcitabine-squalene with biomembranes was improved compared to free gemcitabine [11;12]. Previously results also were obtained by Sarpietro *et al.* [273] with squalene-acyclovir. These authors observed higher absorption into artificial membranes and suggested that the squalene moiety stays in the membrane environment whereas the acyclovir moiety protrudes into the aqueous phase with a small contribution of the prodrug to the phospholipid thermotropic behavior. Recently, a new strategy of squalenoylation was reported [10]. After entering the cell, nucleotide analogues get activated by phosphorylation yielding nucleoside -monophosphate. However, this step is rate-limiting because nucleotide analogues are poor substrates for cellular kinases needed for their activations. To increase efficiency of prodrug, using product of this reaction, nucleoside monophosphate was suggested. However, due to its negatively charged phosphate group nucleoside monophosphates were not able to enter the cell [274]. To solve this problem, squalenoylation of the phosphate moiety was performed creating a lipophilic and amphiphilic molecule which self-assembled in water into particles of 100-300 nm size. These particles were able to penetrate into the cell [10] most likely caused by compact and highly coiled conformation of the squalene moiety in water [275]. As a result for example, nanoassemblies 4-(N)-1,1',2-trisnor-squalenoyldideoxycytidine monophosphate showed better anti-HIV activity and 4-(N)-1,1',2-trisnor-squalenoylgemcitabine monophosphate improved anticancer activity on leukemia cells [10;272;276] compared to nonsqualenoyl analogues.

## Summary and conclusions

In this review article, we summarized our recent knowledge about squalene, an isoprenoid lipid and intermediate of sterol synthesis. Synthesis of squalene is slightly different in microorganisms, plants and mammalian cells. Also utilization of squalene for further metabolic conversion varies in different cell system. In this article, we also addressed biochemical and biophysical properties of squalene. Properties of squalene are unique due to its highly hydrophobic structure. Although cell biological effects of squalene appear to be not dramatic, this lipid may act as a mild modulator of membrane properties.

Recent developments showed that squalene can became a useful component in biotechnology, nutrition and probably in medicine. Therefore, different sources of squalene and processes to isolate this compound became of interest.

It appears that olive oil deodorizer distillate will be used in the future as most efficient squalene source besides shark liver oil. The advantage of olive oil deodorizer distillate is that it consists of relatively high amount of squalene and is a “waste” of olive oil raffination. However, other sources of squalene such as microorganism may also become important. We also describe briefly several biotechnological methods to isolate squalene from different sources. The challenge for these processes is efficiency especially when samples of low squalene concentration are used. The range of squalene utilization in health and nutrition is broad, already. As a biological supplement to the diet and as an additive to drugs it appears to have beneficial properties. Thus, squalene is a versatile molecule which will certainly become a useful compound in biotechnology and pharmacy in the future.

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# Tables

Table 1: **Physical properties of squalene**

| Properties | Values | Ref. |
|---|---|---|
| Octanol/water partitioning coefficient (log P) | 10.67 | [280] |
| Solubility of squalene in water | 0.124 mg/L | [280] |
| Viscosity | ~11 cP | [281-283] |
| Surface tension | ~32 mN/m | [281-283] |
| Density | 0.858 g/mL | [281-283] |

## Figures

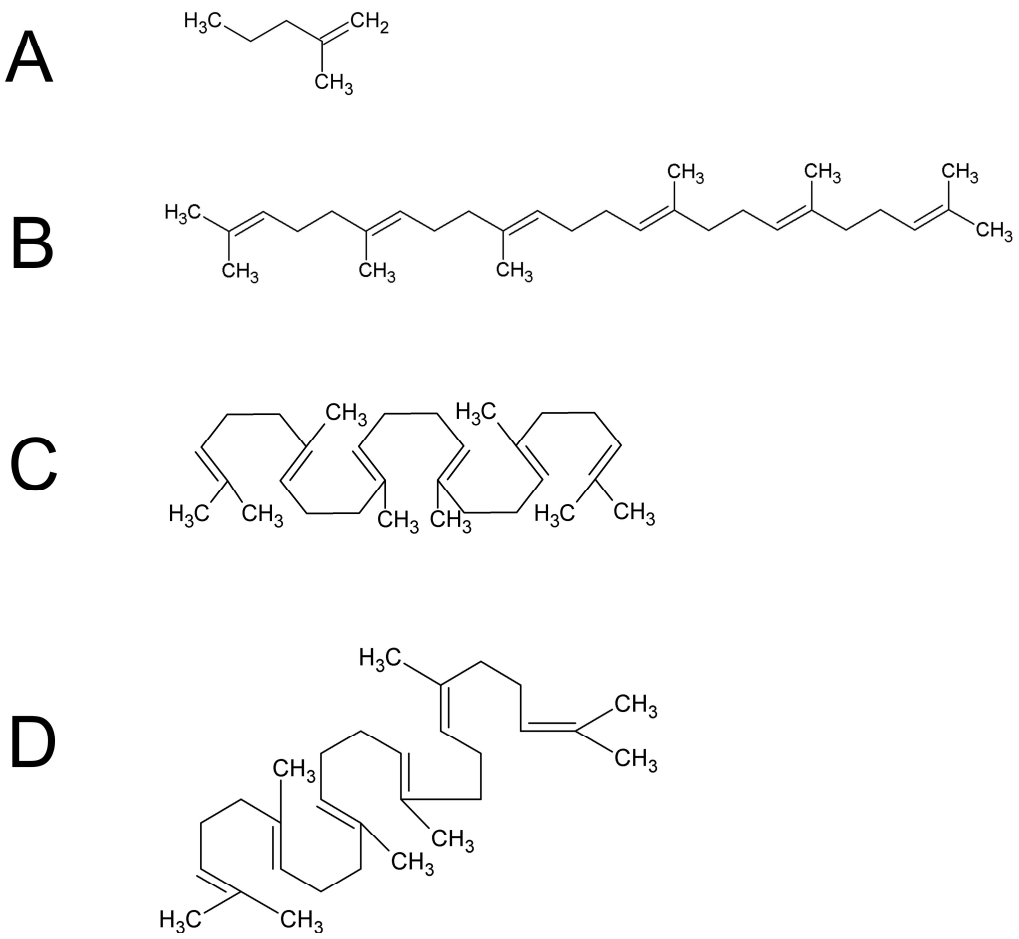


Figure 1. **Chemical structure of squalene and its precursor**

A, Chemical structure of isoprene. Different structures are: B, stretched form; C, coiled form; and D, “sterol-like” form

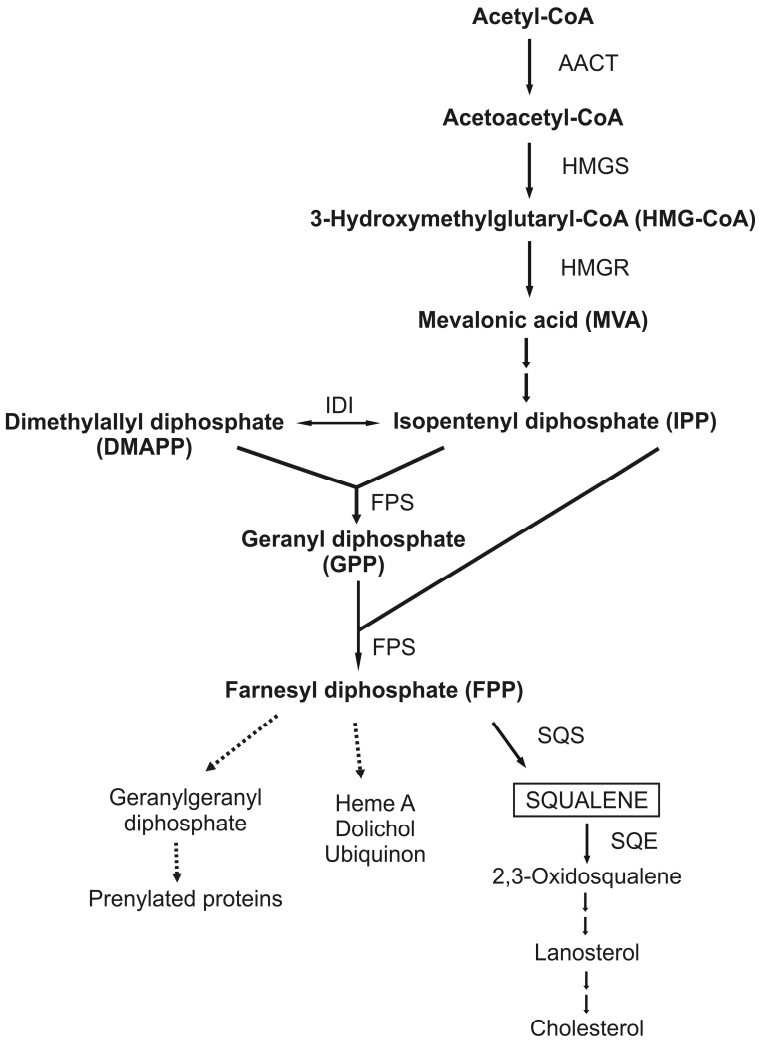


Figure2. **Squalene synthesis via mevalonate pathway in mammalian, yeast and microalgae cells**

AACT, acetoacetyl-CoA thiolase; FPS, FPP synthase; HMGS, HMG-CoA synthase; HMGR, HMG-CoA reductase; IDI, isopenthenyl diphosphate isomerase; SQS, squalene synthase; SQE, squalene epoxidase.

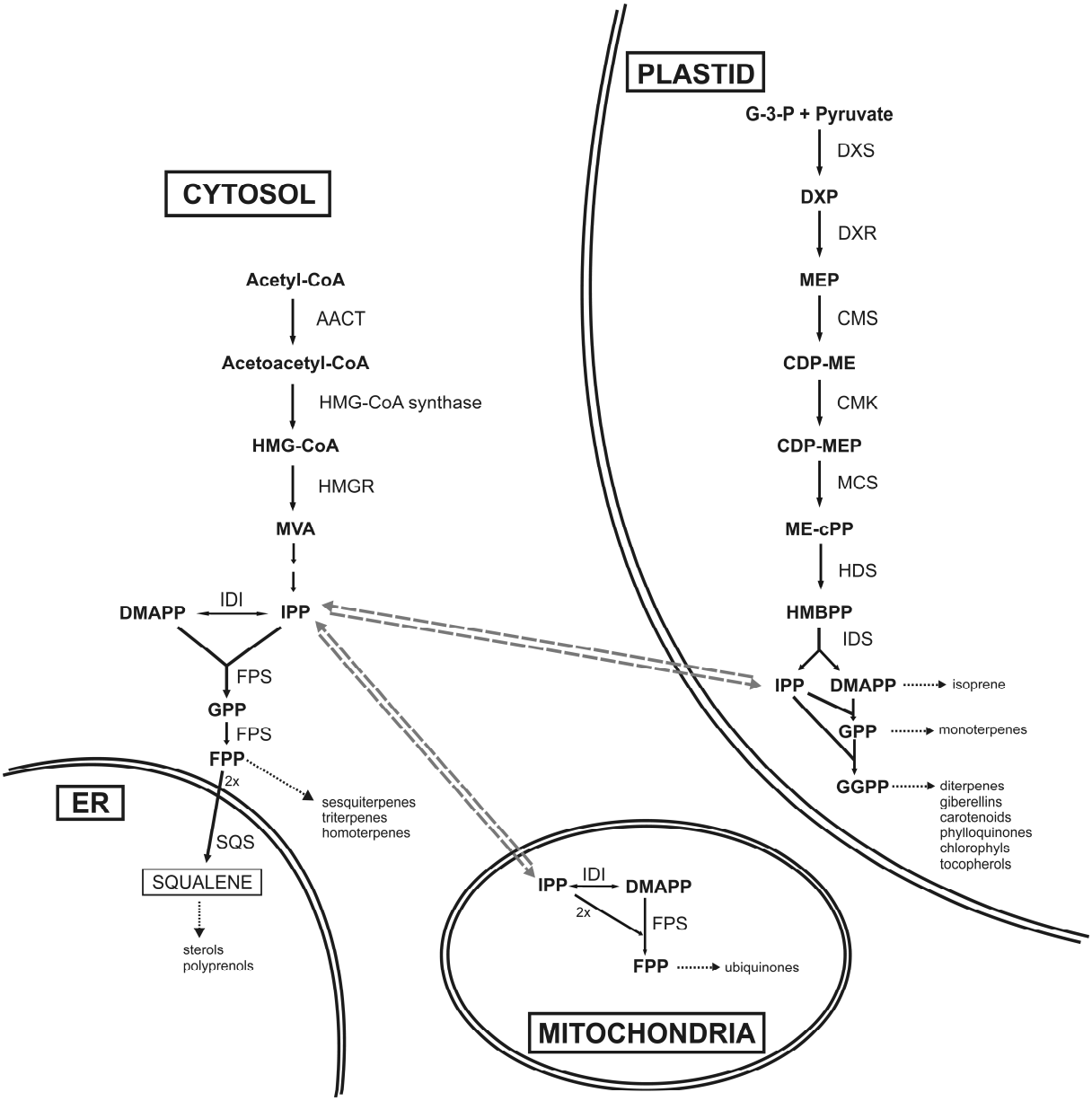


Figure 3. **Squalene synthesis in plants via mevalonate (MVA) pathway in cytosol and methylerythriol phosphate (MEP) pathway in plastids**

The product of MVA pathway, IPP is further metabolized into FPP. FPP either forms sterols and polyprenols via squalene in the ER or is metabolized to sesquiterpenes, triterpenes and homoterpenes. In mitochondria IPP condensates with DMAPP yielding ubiquinons. MEP pathway products are monoterpenes, diterpenes, tocopherols, carotenoids etc. in plastids. Updated and simplified from [277].

AACT, acetoacetyl-CoA thiolase; CDP-ME, 4-(cytidine 5′-diphospho)-2-*C*-methyl-d-erythritol; CDP-MEP, 4-(cytidine 5′-diphospho)-2-*C*-methyl-d-erythritol phosphate;CMK, 4-(cytidine 5′-diphospho)-2-*C*-methyl-D-erythritol kinase; CMS, 4-(cytidine 5′-diphospho)-2-*C*-methyl-D-erythritol synthase; DMAPP, dimethylallyl diphosphate; DXP, 1-deoxy-D-xylulose-5-phosphate; DXR, DXP reductoisomerase; DXS, DXP synthase; FPP, farnesyl diphosphate; FPS, FPP synthase; G-3-P, Glycerol-3-phosphate; GPP, geranyl diphosphate; GGPP, geranylgeranyl diphosphate; HDS, 4-hydroxy-3-methylbut-2-enyl diphosphate synthase; HMBPP, 4-hydroxy-3-methylbut-2-enyl diphosphate; HMG-CoA, 3-hydroxy-3-methyl-glutaryl-CoA; HMGS, HMG-CoA synthase; HMGR, HMG-CoA reductase; IDI, isopenthenyl diphosphate isomerase; IDS, IPP/DMAPP synthase, 4-hydroxy-3-methylbut-2-enyl diphosphate reductase; IPP, isopentenyl diphosphate; MCT, 2-*C*-methyl-d-erythritol 4-phosphate cytidylyltransferase; MDS, 2-*C*-methyl-d-erythritol 2,4-cyclodiphosphate synthase; ME-cPP, 2-*C*-methyl-d-erythritol 2,4-cyclodiphosphate; MEP, 2-C-methyl-D-erythritol 4-phosphate; MVA, mevalonate; SQS, squalene synthase; SQE, squalene epoxidase; ER, endoplasmic reticulum.

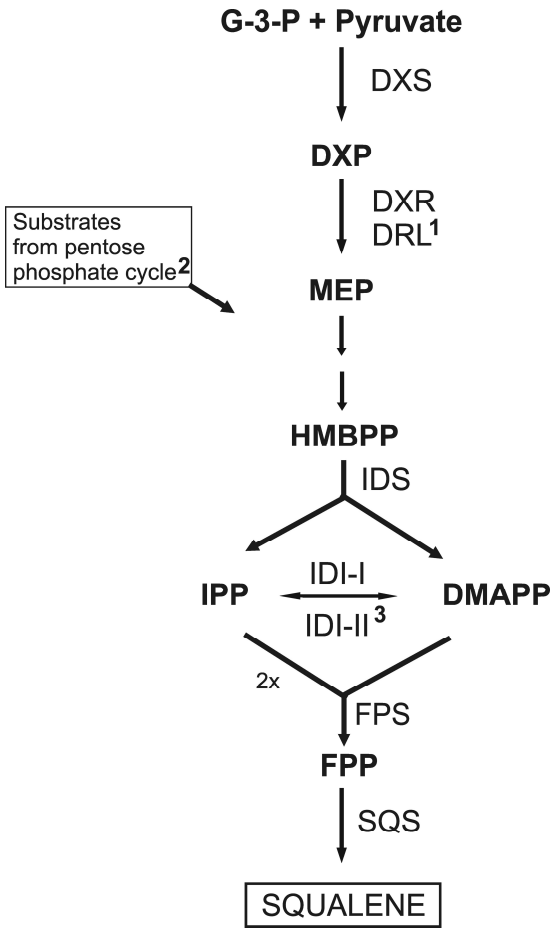


Figure 4. **Squalene synthesized via methylerythritol phosphate (MEP) pathway in *E. coli*.** DXS, DXP synthase; DXR, DXP reductoisomerase; FPS, FPP synthase; SQS, squalene synthase

[1] DRL, DXR-Like protein found in *B. abortus* [129]

[2] Cyanobacteria can utilize substrates from pentose phosphate cycle derived from photosynthesis [278].

[3] IPP isomerase type II was found in cyanobacteria [279] and in actinomycetes *Streptomyces sp.* (in MVA pathway) [132].

Chapter 2

# Effect of Lipid Particle Biogenesis on the Subcellular Distribution of Squalene in the Yeast *Saccharomyces cerevisiae*

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Abbreviations: SE: steryl ester(s); TAG: triacylglycerol(s); QM: quadruple mutant

Running title: Squalene storage in yeast

## Abstract

Squalene belongs to the group of isoprenoids and is a precursor for the synthesis of sterols, steroids and ubiquinons. In the yeast *Saccharomyces cerevisiae* the amount of squalene can be increased by variation of growth conditions or by genetic manipulation. In this report we show that a *hem1*Δ mutant accumulated a large amount of squalene which was stored almost exclusively in cytoplasmic lipid particles/droplets. Interestingly, a strain bearing a *hem1*Δ deletion in a *dga1Δlro1Δare1Δare2Δ* quadruple mutant background (QM*hem1Δ*) which is devoid of the classical storage lipids, triacylglycerols (TAG) and steryl esters (SE), and lacks lipid particles accumulated squalene at similar amounts as the *hem1*Δ mutant in wild type background. In QM*hem1Δ*, however, increased amounts of squalene were found in cellular membranes, especially in microsomes. The fact that QM*hem1Δ* did not form lipid particles indicated that accumulation of squalene solely was not sufficient to initiate proliferation of lipid particles. Most importantly, these results also demonstrated that (i) squalene was not lipotoxic under the conditions tested; and (ii) organelle membranes in yeast can accommodate relatively large quantities of this non-polar lipid without compromising cellular functions. In summary, localization of squalene as described here can be regarded as an unconventional example of non-polar lipid storage in cellular membranes.

# Introduction

During the last decades, the search for novel drugs and compounds used in biotechnology led to an increased interest in natural products with specific properties. One of the substances detected in biological screenings was squalene, an intermediate in sterol biosynthesis. Squalene is a natural compound which belongs to the group of isoprenoids and is precursor for the synthesis of sterols, steroids and ubiquinons. It is one of the most important lipids in skin cells where it secures beneficial mechanical properties of the skin, e.g., skin hydratation [1]. Squalene also belongs to the family of antioxidants [2;3] and was shown to possess antilipidemic and membrane-modulating properties [4;5]. It has also been reported for treatment of skin disorders, cancer, cardiac ailments and liver diseases [6;7]. Presently, squalene used for commercial purposes is isolated from shark liver oil or olive oil. However, alternative biotechnological systems became attractive which may lead to high yield production of this non-polar lipid. As one of these systems the yeast *Saccharomyces cerevisiae* was considered.

In the yeast, squalene is synthesized in a sequence of reactions starting from acetyl-CoA involving a number of steps catalyzed by Erg-proteins [8]. The squalene synthase Erg9p converts farnesyl diphosphate to squalene in an NAD(P)H dependent reaction. Erg9p is localized to the endoplasmic reticulum. In the following oxygen- dependent reaction, the squalene epoxidase Erg1p catalyzes formation of 2,3-oxidosqualene (Fig. 1). Erg1p was shown to be dually located within the yeast cell, namely in lipid particles and in the microsomal fraction [9]. Interestingly, the enzyme from lipid particles did not exhibit activity *in vitro* indicating that additional components of the endoplasmic reticulum were required. The next step in the sterol biosynthetic route is catalyzed by the oxidosqualene cyclase (lanosterol synthase) Erg7p which forms lanosterol, the first sterol in this pathway. Lanosterol is then further converted in several steps to the yeast-specific ergosterol.

The process of sterol biosynthesis is strictly aerobic not only because of the direct oxygen requirement for squalene epoxidation catalyzed by Erg1p but also due to the involvement of heme (synthesized in an oxygen-dependent manner) in several steps in ergosterol biosynthesis. As an example, heme is essential for the activity of the sterol-14-α-demethylase Erg11p (see Fig. 1), an NADPH-heme-dependent cytochrome P450 protein [10;11]. Consequently, yeast cells become auxotrophic for sterols and unsaturated fatty acids (UFA) when grown anaerobically or under

heme deficiency. To warrant cell viability under such conditions, sterols and UFA have to be supplemented to the growth medium [12-15]. When yeast cells are unable to synthesize ergosterol in the absence of oxygen or due to heme deficiency, squalene accumulates. Other strategies to cause a cellular increase of squalene in the yeast are overexpression of *HMG1* [16;17] and disruption of *ERG1* or *ERG7* [18;19].

The aim of the present study was to investigate localization of squalene in yeast cells especially under conditions which stimulated accumulation of this non-polar lipid. For this purpose, we used cells bearing a deletion of the *HEM1* gene in wild type background. Previous studies from our laboratory [20] had already suggested the presence of squalene in lipid particles. Since lipid particles serve as storage compartment for neutral lipids like triacylglycerols (TAG) or steryl esters (SE), this compartment appeared to be the "logical" depot for the excess of squalene as well. In the present work, however, we extended our investigations to the subcellular distribution of squalene in cells deprived of lipid particles. This situation occurs in a *dga1Δlro1Δare1Δare2Δ* quadruple mutant (QM) which lacks the four enzymes catalyzing TAG and SE formation in the yeast [21-23]. The QM grows like wild type under standard conditions but is compromised when fatty acids are present in the medium [24;25] or used as a carbon source (our own unpublished results).

This cellular scenario of the QM was considered to be highly relevant for possible lipotoxic effects of squalene when accumulated as a consequence of various manipulations. As will be shown in this work, obvious lipotoxic effects of squalene were avoided in a QM*hem1*Δ strain by incorporation of this compound into subcellular membranes. This finding was surprising and has never been described in the literature for biological membranes, although squalene incorporation into artificial membranes has been reported before [26;27].

## Experimental procedures

*Strains, culture conditions and subcellular fractionation*

Yeast strains used in this study are listed in Table 1. Inactivation of the *HEM1* gene was performed by using the pUC19 plasmid which contains a *hem1::LEU2* disruption cassette. The plasmid was cleaved using the restriction enzymes BamHI and HindIII, and the disruption cassette was transformed into yeast cells by standard procedures. The disruption of *HEM1* in mutants was confirmed by colony PCR.

Cells were grown aerobically to the early or late stationary phase at 30°C in YPD medium containing 1% yeast extract (Oxoid), 2% peptone (Oxoid) and 2% glucose (Merck). Media were inoculated with precultures to the $OD_{600}$ of 0.1. Strains deleted of *HEM1* were grown on YPD medium supplemented with either 50 μg/ml δ-aminolevulinic acid (δALA) or 20 μg/ml ergosterol (Erg) with 0.06% Tween 80 as a source of oleic acid.

The yeast lipid particle fraction was obtained at high purity from cells grown to the stationary phase as described by Leber *et al.* [20]with an additional washing step with 9 M urea [28]. Isolation of other subcellular fractions, e.g., mitochondria, heavy and light microsomes and plasma membrane used in this study was described by Zinser *et al.* [29]. The quality of subcellular fractions was routinely tested by Western blot analysis (see below).

*Protein analysis*

Proteins were quantified by the method of Lowry *et al.* [30] using bovine serum albumin as a standard. Polypeptides were precipitated with trichloroacetic acid and solubilized in 0.1% sodium dodecyl sulfate (SDS), 0.1 M NaOH prior to quantification. Samples of the lipid particle fractions were delipidated prior to protein quantification. Non-polar lipids were extracted with 2 volumes of diethyl ether, the organic phase was withdrawn, residual diethyl ether was removed under a stream of nitrogen, and proteins were precipitated as described above.

SDS-polyacrylamide gel electrophoresis was carried out by the method of Laemmli [31]. Samples were denaturated at 37°C to avoid hydrolysis of polypeptides. Proteins on gels were detected by staining with Commassie Blue. Western blot analysis was performed as described by Haid and Suissa [32]. Proteins were detected by enzyme-linked immunosorbent assay (ELISA) using rabbit antisera as primary antibody and peroxidase-conjugated goat anti-rabbit IgG as

second antibody. Primary antibodies used in this study were from rabbits and directed against yeast Erg6p, Por1p, Wbp1p and Gas1p. The enhanced chemiluminescent signal detection kit SuperSignal™ (Pierce Chemical Company, Rockford, IL, USA) was used to visualize immunoreactive bands which were quantified densitometrically.

*Lipid analysis*

Lipids from yeast cells were extracted as described by Folch *et al.* [33]. For quantification of neutral lipids, extracts were applied to Silica Gel 60 plates with the aid of a sample applicator (CAMAG, Automatic TLC Sampler 4, Muttenz, Switzerland), and chromatograms were developed in an ascending manner by a two step developing system. First, light petroleum:diethyl ether:acetic acid (70:30:2; per vol.) was used as mobile phase, and plates were developed to half distance of the plate. Then, plates were dried briefly and further developed to the top of the plate using the second mobile phase consisting of light petrolum:diethyl ether (49:1; v/v). To visualize separated bands, TLC plates were dipped into a charring solution consisting of 0.63 g MnCl2 x 4H2O, 60 ml water, 60 ml methanol and 4 ml concentrated sulphuric acid, briefly dried and heated at 100°C for 30 min. Then, lipids were quantified by densitometric scanning at 400 nm using a Shimadzu dual-wave length chromatoscanner CS-930 with triolein and cholesteryl ester as standards.

Squalene and individual sterols (free sterols and SE) from whole cells or subcellular fractions were identified and quantified by GLC-MS [34]. GLC-MS was performed on an HP 5890 Gas-Chromatograph equipped with a mass selective detector HP 5972, using an HP5-MS capillary column (30 m x 0.25 mm, 0.25 µm film thickness). Aliquots of 1 µl were injected in the splitless mode at 270°C injection temperature with helium as a carrier gas at a flow rate of 0.9 ml/min in constant flow mode. The following temperature program was used: 1 min at 100°C, 10°C/min to 250°C, and 3°C/min to 310°C. Mass spectra were acquired in the scan mode (scan range 200-550 amu) with 3.27 scans per second. Sterols were identified based on their mass fragmentation pattern.

*Fluorescence microscopy of lipid particles*

Nile Red staining was performed as described by Greenspan *et al.* [35]. Microscopic pictures were taken with an Olympus BX50 photomicroscope equipped with a color digital camera DP70 (Olympus) using a filter U-MWU (Dichroic mirror: DM570, Exciter filter: BP480-550, Barrier filter: BA590). Nile Red fluorescence of lipid particles was detected at an emission wavelength of 590 nm. Digital color pictures were transformed to grey scale figures by standard software.

*Electron microscopy of yeast cells*

For ultrastructural inspection, cells were grown under aerobic conditions at 30°C on YPD with or without supplementations as described above. Cells were harvested in the early stationary phase by centrifugation and washed 3 times with bidest. water. Subsequently, cells were fixed for 5 min in a 1% aqueous solution of $KMnO_4$ at room temperature, washed with dist. water and fixed in a 1% aqueous solution of $KMnO_4$ for 20 min again. Fixed cells were washed three times in dist. water and incubated in 0.5 % aqueous uranylacetate overnight at 4°C. Samples were dehydrated in a graded series of acetone (50, 70, 90 and 100 %) and gradually infiltrated with increasing concentrations of Spurr resin (30%, 50%, 70% and 100%) mixed with acetone for a minimum of 3h for each step. Samples were finally embedded in pure, fresh Spurr resin and polymerized at 60°C for 48 h. Ultrathin sections of 80 nm were stained with lead citrate and viewed with a Philips CM 10 electron microscope.

## Results

*Heme-deficient cells accumulate squalene*

Disruption of *HEM1* encoding δ-aminolevulinic acid synthase leads to cessation of the ergosterol pathway at the level of lanosterol demethylation (see Fig. 1). Consequently, *hem1*Δ mutants accumulated lanosterol (Fig. 2A), but also showed an approximately 300-fold increase of squalene. This accumulation of squalene resulted in a marked change of the neutral lipid pattern. Quantification of neutral lipids after TLC separation revealed that in wild type the ratio of squalene to SE and TAG, respectively, was close to zero whereas it increased to 1.7 (for SE) and 0.88 (for TAG) in the *hem1*Δ mutant. It has to be noted that ergosterol (free and acylated form) detected in *hem1Δ* strains was derived either from exogenous sterol supplementation or through bypassing the defect by supplementation of δALA to the growth medium [14;15] which restored heme synthesis (see Fig. 2A). Under the latter conditions the sterol pattern was close to wild type grown with or without supplements (data not shown), and squalene accumulation was not observed.

The QM *dga1Δlro1Δare1Δare2Δ*, which lacks enzymes required for the biosynthesis of TAG and SE, exhibited almost the same sterol pattern as wild type (Fig. 2B). *HEM1* deletion in the QM background led also to a huge increase of squalene. Since QM*hem1Δ* was unable to synthesize TAG and SE, the accumulated squalene encountered for the major non-polar lipid species in this strain. This observation led us to investigate whether or not this large amount of squalene was sufficient and capable of initiating the formation of lipid storage particles.

*Squalene alone does not initiate lipid particle formation*

As described above, squalene accumulated in heme-deficient cells. The question remained where squalene was localized under these conditions. In wild type cells, a small amount of squalene (0.5 % of total mass) has been detected in lipid particles [20;36]. Therefore, it was conceivable that squalene accumulating in a *hem1Δ* mutant would also be preferentially found in this lipid storage compartment. Since lipid particles have been considered as a depot for excess fatty acids which may be toxic for the cell [37], we anticipated that the same storage mechanism may also apply to squalene.

To address this question we performed fluorescent microscopy of cells stained with the fluorescent dye Nile Red. This dye is specific for neutral lipids and has been widely used to visualize lipid particles/droplets. As expected, lipid particles were observed in wild type and in a *hem1Δ* deletion mutant (Figs 3A and B). Thus, the usual lipid storage compartment was present in these cells, but the localization of squalene could not be attributed by this method. In accordance with published observations, lipid particles were missing in QM (Fig. 3C). The striking result, however, was that in the squalene accumulating QM*hem1Δ* strain lipid particles or lipid droplet-like structures were not detected either (Fig. 3D). This result could be interpreted in two different ways. First, lipid particles may indeed not be formed in QM cells even under conditions promoting squalene accumulation. Secondly, squalene and consequently squalene-loaded lipid particles might not be properly visualized with Nile Red. To address the latter question, we subjected the same cells to electron microscopy. As can be seen from Fig. 4, the results paralleled completely the fluorescence microscopy analysis. Whereas lipid particles were detected in wild type and *hem1Δ*, no such structures were observed in the QM background. Most notably, the QM*hem1Δ* strain did not contain lipid particle structures indicating that squalene accumulation in the absence of TAG and SE was not sufficient to induce lipid particle proliferation. This view was supported by the fact that all our attempts to isolate lipid particles from strains with QM background failed. These results together with data shown in Fig. 2 also suggested that under these conditions squalene was obviously localized to other subcellular sites.

*Subcellular distribution of squalene in wild type and in the quadruple mutant dga1Δlro1Δare1Δare2Δ*

To obtain a deeper insight into the subcellular distribution of squalene in the different strains mentioned above, mitochondria, 30,000 *g*, 40,000 *g* and 100,000 *g* microsomes, lipid particles, plasma membrane and cytosol were isolated from wild type, *hem1Δ*, QM and QM*hem1Δ* and subjected to detailed analysis.

First, all isolated subcellular fractions were tested for purity by Western Blotting. Enzyme markers for plasma membrane (Gas1p), mitochondria (Por1p), microsomes (Wbp1p) and lipid particles (Erg6p) were used. As shown in Table 2, all organelles from the different strains were obtained at the expected quality in line with previously published results [29]. In all strains, plasma membrane and lipid particles were obtained at high enrichment, and mitochondria and

microsomal fractions were obtained at good quality with acceptable cross-contamination. It has to be mentioned, however, that in *hem1Δ* and QM*hem1Δ* contamination between microsomes and mitochondria was higher that in wild type and QM. This problem could not be overcome by a number of modifications in the isolation protocol and appears to be an intrinsic property of these organelles under heme depletion.

The subcellular distribution of squalene and ergosterol in wild type and mutant strains is shown in Table 3, and the enrichment of these components in the individual organelles from the different strains is documented in the Supplementary Figures S1-S4. In wild type, the highest amount of ergosterol was detected in microsomal and mitochondrial compartments (see Table 3) although the highest enrichment was found in the plasma membrane (Fig. S1A). Squalene was present mainly in lipid particles and microsomes. The observation that squalene accumulated in lipid particles from wild type (106 mg/g organelle protein; see Fig. S1B) was in line with previous findings [20;36]. In the lipid particle fraction ergosterol, lanosterol and other sterol precursors (not shown) were also enriched. It has to be noted that the large majority of sterols in lipid particles occurs in the acylated form as SE.

In the QM, sterols were found mainly in mitochondria and microsomes (see Table 3 and Fig. S2). Noteworthy, the enrichment of ergosterol in the plasma membrane was not as high as in wild type, whereas larger concentrations of ergosterol were detected in microsomes and also in mitochondria. Due to the quadruple deletions of *DGA1, LRO1, ARE1* and *ARE2* lipid particles were missing in this strain. Similar to ergosterol, squalene was found in microsomal and mitochondrial membranes of the QM. The enrichment of squalene in microsomes and mitochondria from the QM was higher than in wild type, but still moderate. The slight accumulation of squalene in QM may be result of reduced formation of squalene epoxidase (Erg1p) in this strain [23].

*Subcellular distribution of squalene in hem1Δ and in* QM*hem1Δ*

Yeast strains deleted of *HEM1* bear defects in the sterol biosynthetic pathway and fatty acid desaturation. To maintain growth of such mutants, the respective supplements in the media need to be provided (see Experimental Procedures). As a consequence of this defect, *hem1Δ* mutants formed substantial amounts of squalene and lanosterol (see Fig. 2). Based on previous findings [20;36] we assumed that under these conditions large amounts of squalene accumulated

in lipid particles. As can be seen from Table 3 this was correct. Whereas in other subcellular membranes ergosterol (imported from the medium) was still the major sterol, and squalene was present only at smaller concentrations (Fig. S3A), the level of squalene in lipid particles of *hem1Δ* by far exceeded that of ergosterol (Fig. S3B). This finding was not surprising because of the low esterification efficiency of external ergosterol as reported by Valachovic *et al.* [38].

In Fig. 2 it is shown that a QM deleted of *HEM1* accumulated squalene at similar amounts as *hem1Δ* in the wild type background. Since the QM*hem1Δ* strain did not contain lipid particles (see Figs 3 and 4) it was assumed, that squalene might be localized to other organelles. Indeed, marked amounts of squalene were detected in mitochondrial and microsomal fractions (see Table 3, and Fig. S4). This subcellular distribution of squalene reflected very much the localization of ergosterol and lanosterol in these cells. Due to the cross-contamination of mitochondria and microsomes (see Table 2) in QM*hem1Δ* compared to QM and wild type the absolute amount of squalene and sterols present in these fractions has to be interpreted with caution. The fact, however, remains that under heme depletion in a QM background intracellular membranes were able to accommodate large amounts of the non-polar lipid squalene.

## Discussion

For the work presented here we created a situation in the cell caused by accumulation of squalene that we considered lipotoxic. For this purpose, we used the yeast *Saccharomyces cerevisiae* bearing a deletion of the *HEM1* gene. Such *hem1*Δ strains have been widely used as a model for anaerobic growth [39;40] because lack of cytochromes results in metabolic changes similar to oxygen deficiency. It has, however, also been shown that such *hem1*Δ strains can grow reasonably well as long as ergosterol and unsaturated fatty acids are supplied from the medium [14;15].

In the present study we show that squalene overproduced under heme deficiency in wild type cells mainly ends up in lipid particles. This was expected because this compartment is considered a depot or even a sink for storage lipids produced in excess, e.g., fatty acids. The real challenge for the yeast cell arises when lipid particles are missing. This situation occurs in a heme deficient quadruple mutant QM*hem1Δ*. To our surprise, these cells were viable although accumulating substantial amounts of squalene indicating that squalene was not lipotoxic, at least under conditions chosen and at the amounts accumulated.

We considered the findings described above very important for two more reasons: (i) Squalene, similar to TAG and SE, is formed in the ER [41]. In previous studies from our lab [22;23] we had found that synthesis of either TAG or SE was sufficient to promote the biosynthesis of lipid particles. A model widely advocated for lipid particle biogenesis suggests that neutral lipid synthesized in the ER may be the sparking point for lipid particle biogenesis [22]. This may, however, be true for TAG and SE, but according to our results not for squalene. (ii) Since the QM*hem1Δ* mutant strain did not contain lipid particles but nevertheless accumulated large amounts of squalene, this component had to be accommodated in organelle fractions different from lipid particles. As already observed in the *hem1Δ* mutant strain in wild type background, squalene of QM*hem1Δ* was detected in mitochondria and microsomes, but at much higher amounts.

Localization of squalene in biological membranes existing mainly as phospholipid bilayers is obviously not a regular situation, because due to its shape, the high hydrophobicity and the lack of a polar head group squalene cannot be expected to form bilayer structures *per se*. However, localization of squalene in the membranes may be explained by observations of Lohner *et al.* [26],

who studied the influence of squalene on artificial membranes. These authors concluded that (i) squalene at a concentration of 6 mol % in phospholipid vesicles changed the lamellar-to-inverse-hexagonal phase transition by increasing the size of the tubes of the inverse hexagonal phase; and (ii) squalene must be accommodated in a most disordered region of the bilayer, suggesting that squalene rather exists in a coiled than extended conformation and localizes to the interior of the bilayer. The localization of polyisoprene hydrocarbons in the midplane of a lipid bilayer was also reported by Hauss *et al.* [27]. These authors studied the role of squalane (saturated form of squalene) in artificial phospholipid membranes by neutron diffraction. They argued that this specific membrane structure may function as a proton permeability barrier [42]. This may also apply to biological membranes, because yeast cells accumulating squalene in membranes appear to exhibit altered sensitivity to low pH and high salt concentrations compared to wild type (our own preliminary and unpublished results).

The question remains how squalene synthesized in the endoplasmic reticulum reaches its different cellular destinations. In the case of storage in the lipid particles a co-migration with TAG and SE in the course of lipid particle biogenesis may be anticipated. Transport of squalene to other organelles and incorporation into membranes may involve other mechanisms generally discussed for lipid transport and assembly such as protein mediated transport, vesicle flux or membrane contact [43;44]. In summary, subcellular distribution of squalene as shown in this study can be regarded as a novel facet of lipid storage in membranes and shows at the same time the high flexibility of the yeast as a model system to adapt to lipid stress situations.

## Acknowledgements

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# Tables

Table 1. **Yeast strains used in this study**

| Yeast strains | Genotype | Source or Ref. |
|---|---|---|
| Wild type BY4742 | *MATa ; his3D1; leu2D0; lys2D0; ura3D0* | Euroscarf |
| QM *dga1Δ lro1Δ are1Δ are2Δ* | *MATa ; his3D1; leu2D0; lys2D0; ura3D0; dga1::KanMX4; lro1::KanMX4; are1::KanMX4; are2::KanMX4* | K. Athenstaedt |
| *hem1Δ* | *MATa ; his3D1; leu2D0; lys2D0; ura3D0; hem1::LEU2* | This study |
| QM*hem1Δ* | *MATa ; his3D1; leu2D0; lys2D0; ura3D0; dga1::KanMX4; lro1::KanMX4; are1::KanMX4; are2::KanMX4; hem1::LEU2* | This study |

Table 2. **Quality control of yeast subcellular fractions**

For growth conditions and the isolation of subcellular compartments see Experimental Procedures section. The relative enrichment of markers in the homogenate was set as 1. Marker proteins: Gas1p, β-1,3-glucanosyltransferase (marker for plasma membrane); Wbp1p, β-subunit of the oligosaccharyl transferase glycoprotein complex (marker for microsomes); Por1p, porin (marker for mitochondria); Erg6p, Δ(24)-sterol C-methyltransferase (marker for lipid particles). n.d.: not detectable.

| | Relative enrichment (fold) | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | **wild type** | | | | ***hem1Δ*** | | | | **QM** | | | **QM*hem1Δ*** | | |
| | Gas1p | Wbp1p | Por1p | Erg6p | Gas1p | Wbp1p | Por1p | Erg6p | Gas1p | Wbp1p | Por1p | Gas1p | Wbp1p | Por1p |
| Mitochondria | n.d. | 1.0 ± 0.66 | 3.8 ± 1.5 | n.d. | n.d. | 1.0 ± 0.40 | 3.2 ± 0.91 | 0.6 | n.d. | 1.5 ± 0.75 | 2.3 ± 0.43 | 2.0 | 2.1 ± 0.043 | 2.0 ± 0.50 |
| Microsomes 30.000 *g* | n.d. | 2.5 ± 0.38 | 0.5 ± 0.2 | n.d. | n.d. | 1.3 ± 0.24 | 0.1 ± 0.09 | 0.4 | n.d. | 3.0 ± 0.40 | n.d. | 1.0 | 2.9 ± 0.96 | 1.2 ± 0.20 |
| Microsomes 40.000 *g* | n.d. | 1.9 ± 0.075 | n.d. | n.d. | n.d. | 1.1 ± 0.24 | n.d. | n.d. | n.d. | 2.8 ± 0.96 | n.d. | n.d. | 1.1 ± 0.53 | 0.2 ± 0.058 |
| Microsomes 100.000 *g* | n.d. | 0.7 ± 0.001 | n.d. | n.d. | n.d. | 0.3 ± 0.2 | n.d. | n.d. | n.d. | n.d. | n.d. | n.d. | n.d. | 0.1 ± 0.08 |
| Cytosol | n.d. | n.d. | n.d. | n.d. | n.d. | n.d. | n.d. | n.d. | n.d. | n.d. | n.d. | n.d. | n.d. | n.d. |
| Plasma membrane | ~100 | 1.2 ± 0.14 | 0.3 ± 0.07 | n.d. | ~100 | 2.5 ± 1.1 | 2.7 ± 1.2 | n.d. | ~100 | 2.3 ± 1.1 | 2.5 ± 0.78 | ~100 | 1.1 ± 0.66 | 1.2 ± 0.75 |
| Lipid particles | n.d. | 1.5 ± 0.75 | 0.5 ± 0.3 | ~100 | n.d. | 1.0 ± 0.60 | 0.5 ± 0.3 | ~100 | | | | | | |

Table 3. **Quantification of squalene and sterols in organelles.**

Cells were cultivated to the stationary phase with supplements, organelles were isolated as described by Zinser *et al.* [29], proteins were quantified as described by Lowry *et al.* [30] and sterols were analyzed by GLC-MS.

% of homogenate

| | **Wild type** | | ***hem1Δ*** | | | **QM** | | **QM*hem1Δ*** | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | Squalene | Ergosterol | Squalene | Ergosterol | Lanosterol | Squalene | Ergosterol | Squalene | Ergosterol | Lanosterol |
| Mitochondria | n.d. | 4.28 ± 2.28 | 3.07 ± 1.18 | 6.31 ± 2.60 | 8.14 ± 1.43 | 27.56 ± 2.78 | 27.70 ± 5.89 | 17.66 ± 5.18 | 16.59 ± 4.51 | 13.55 ± 0.64 |
| Microsomes 30.000 *g* | 24.79 ± 16.66 | 11.45 ± 2.09 | 9.06 ± 5.04 | 39.80 ± 15.67 | 41.85 ± 10.17 | 33.42 ± 13.75 | 33.41 ± 12.54 | 42.28 ± 5.44 | 46.64 ± 0.69 | 41.59 ± 11.59 |
| Microsomes 40.000 *g* | 7.90 ± 4.59 | 8.76 ± 2.19 | 7.03 ± 1.40 | 22.75 ± 14.32 | 25.29 ± 5.96 | 32.14 ± 15.05 | 33.45 ± 15.45 | 26.69 ± 8.70 | 29.27 ± 5.07 | 30.19 ± 12.18 |
| Microsomes 100.000 *g* | n.d. | 7.39 ± 3.38 | 5.35 ± 2.64 | 21.00 ± 18.89 | 19.72 ± 7.30 | 5.81 ± 1.02 | 2.94 ± 0.35 | 10.81 ± 5.18 | 4.87 ± 2.07 | 12.13 ± 9.24 |
| Cytosol | n.d. | 0.38 ± 0.02 | 0.67 ± 0.24 | 1.68 ± 0.55 | 1.24 ± 0.30 | 0.82 ± 0.83 | 0.95 ± 0.28 | 2.03 ± 0.78 | 1.25 ± 0.36 | 2.06 ± 0.12 |
| Plasma membrane | 0.45 ± 0.31 | 1.35 ± 0.23 | 0.15 ± 0.05 | 1.00 ± 0.05 | 0.95 ± 0.64 | 0.25 ± 0.03 | 1.56 ± 0.02 | 0.54 ± 0.26 | 1.38 ± 0.28 | 0.47 ± 0.31 |
| Lipid particles | 66.86 ± 3.11 | 66.40 ± 2.50 | 74.68 ± 0.37 | 7.47 ± 4.78 | 2.81 ± 2.30 | | | | | |

## Figures

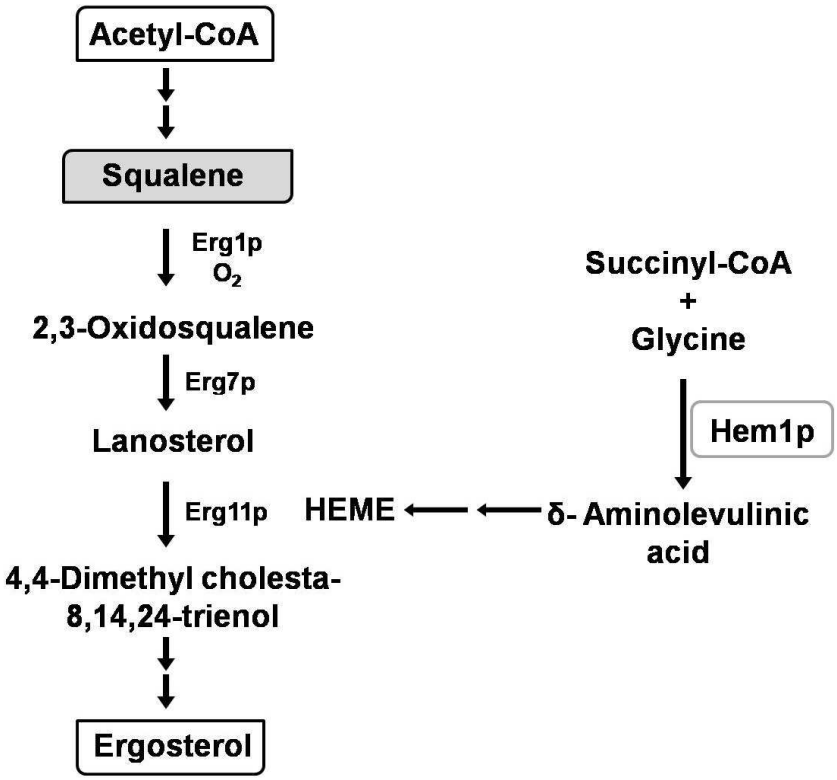


Figure 1: **Heme dependence of ergosterol synthesis.**

The yeast sterol-14-α-demethylase (Erg11p) is a cytochrome P450 protein and depends on heme. Deletion of *HEM1* can be overcome by supplementing yeast cell with δ-aminolevulinic acid (δALA).

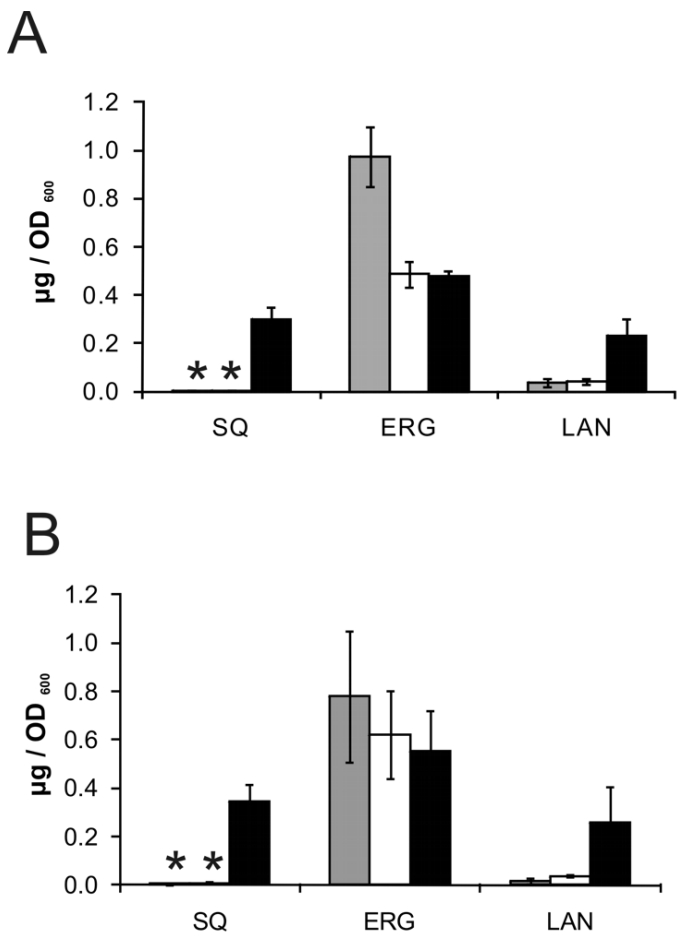


Figure 2: **Sterol composition and squalene accumulation in *hem1Δ* mutants.**

A: Wild type BY4742 (grey bar), *hem1Δ* +δALA (white bar), *hem1Δ* + ergosterol + Tween 80 (black bar), B: Quadruple mutant (QM) *dga1Δlro1Δare1Δare2Δ* (grey bar), QM*hem1Δ* + δALA (white bar), QM*hem1Δ* + ergosterol + Tween 80 (black bar). Cells were grown to the stationary phase, lipids were extracted and sterols/squalene were quantified by GLC-MS. Data are mean values of five independent experiments. Error bars represent the standard deviation. The asterisk (*) indicates that in these samples only marginal amounts of the respective compound were detected. ERG, ergosterol; LAN, lanosterol; SQ, squalene; δALA, δ-aminolevulinic acid.

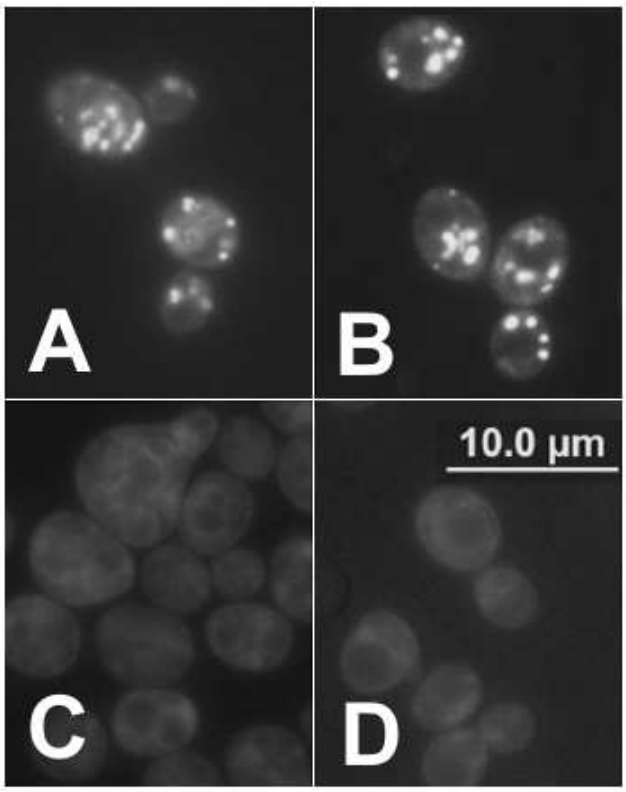


Figure 3: **Visualization of lipid storage compartments by fluorescence microscopy.**
Wild type BY4742 (A), *hem1Δ* (B), QM (C) and QM, *hem1Δ* (D) were grown to the stationary phase, stained with Nile Red and inspected by fluorescent microscope as described in the Experimental Procedures section. Bar: 10 µm.

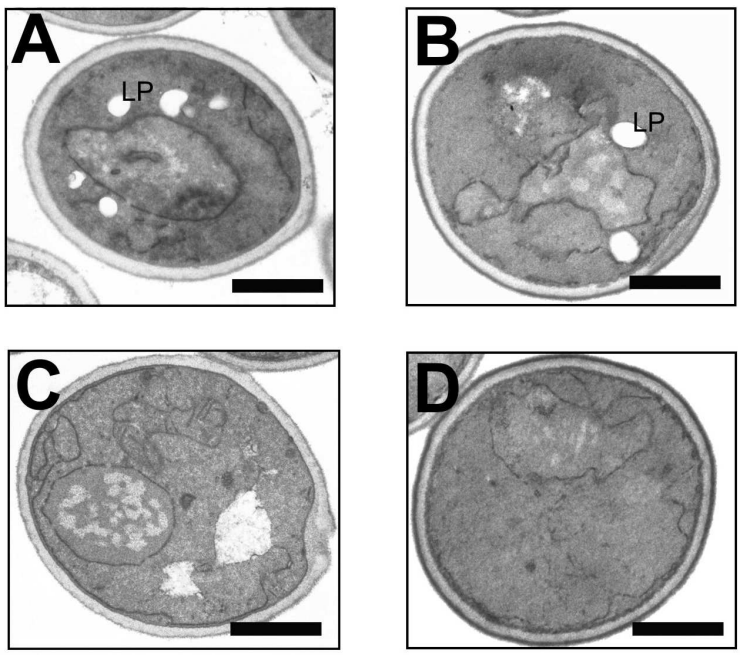


Figure 4: **Overproduction of squalene in a quadruple mutant *dga1Δlro1Δare1Δare2Δ* does not induce lipid particle formation.**

Wild type (A), *hem1Δ* (B), QM (C) and QM*hem1Δ* (D) were grown to the stationary phase and then processed and inspected by electron microscopy as described in the Experimental Procedures section.

Bar: 1 µm. LP, lipid particle.

## Supplemental Figures

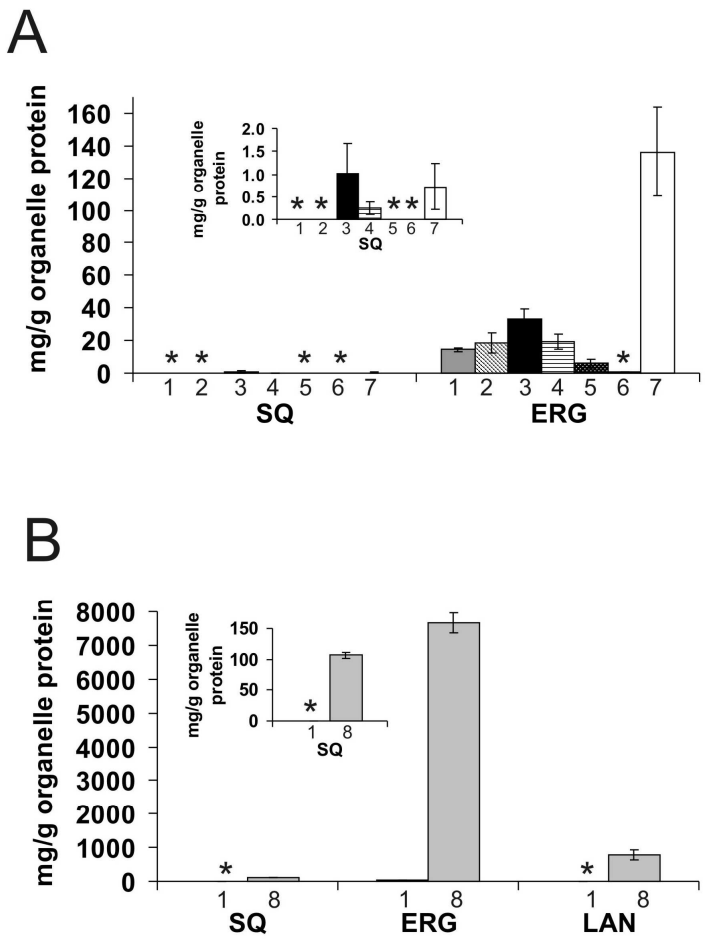


Figure S1: **Quantification of squalene and sterols in organelles from wild type.**

Cells were cultivated to the stationary phase with supplements, organelles were isolated as described by Zinser *et al.* [29], proteins were quantified as described by Lowry *et al.* [30] and sterols were analyzed by GLC-MS. A: 1, homogenate; 2, mitochondria; 3, microsomes 30,000 *g*; 4, microsomes 40,000 *g*; 5, microsomes 100,000 *g*; 6, cytosol; 7, plasma membrane. B: 1, homogenate; 8, lipid particles. The asterisk (*) indicates that in these samples only marginal amounts of the respective compound were detected. Error bars represent the standard deviation. ERG, ergosterol; LAN, lanosterol; SQ, squalene.

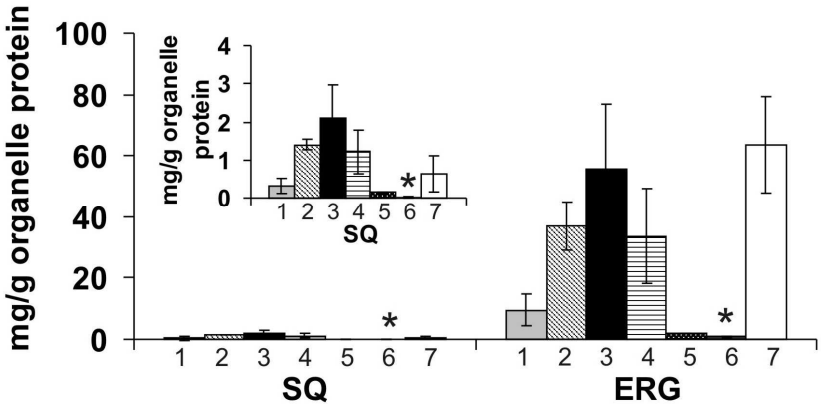


Figure S2: **Quantification of squalene and sterols in organelles from the quadruple mutant (QM) *dga1Δlro1Δare1Δare2Δ*.**

Cells were cultivated and samples were analyzed as described in the legend to Figure S1. 1, homogenate; 2, mitochondria; 3, microsomes 30,000 *g*; 4, microsomes 40,000 *g*; 5, microsomes 100,000 *g*; 6, cytosol; 7, plasma membrane. The asterisk (*) indicates that in these samples only marginal amounts of the respective compound were detected. Error bars represent the standard deviation. ERG, ergosterol; LAN, lanosterol; SQ, squalene.

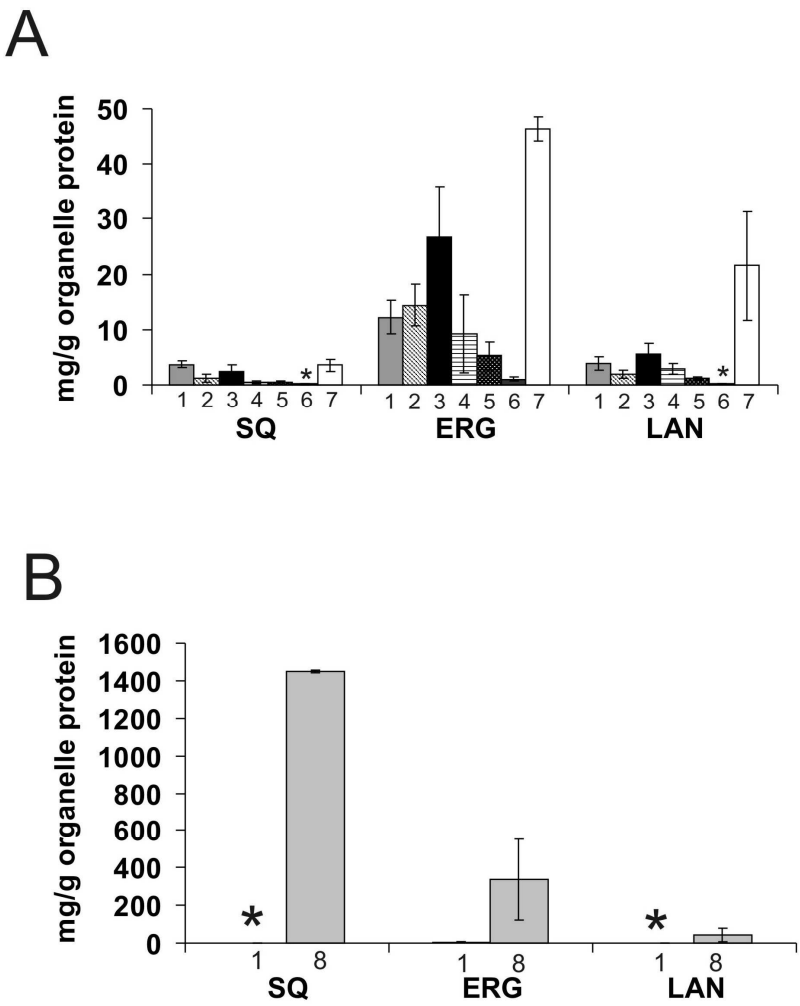


Figure S3: **Quantification of squalene and sterols in organelles from a *hem1Δ* deletion strain.** Cells were cultivated and samples were analyzed as described in the legend to Figure S1. A: 1, homogenate; 2, mitochondria; 3, microsomes 30,000 *g*; 4, microsomes 40,000 *g*; 5, microsomes 100,000 *g*; 6, cytosol; 7, plasma membrane. B: 1, homogenate; 8, lipid particles. The asterisk (*) indicates that in these samples only marginal amounts of the respective compound were detected. Error bars represent the standard deviation. Error bars represent the standard deviation. ERG, ergosterol; LAN, lanosterol; SQ, squalene.

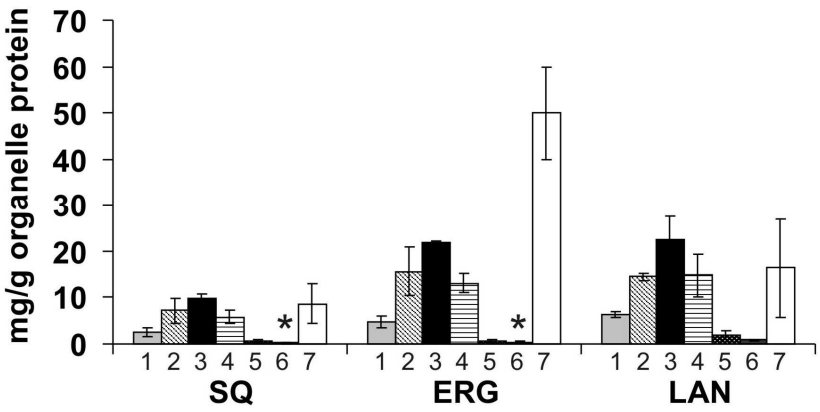


Figure S4: **Quantification of squalene and sterols in organelles from the quadruple mutant (QM) *dga1Δlro1Δare1Δare2Δ* bearing an additional *hem1Δ* deletion.**

Cells were cultivated and samples were analyzed as described in the legend to Figure S1. 1, homogenate; 2, mitochondria; 3, microsomes 30,000 *g*; 4, microsomes 40,000 *g*; 5, microsomes 100,000 *g*; 6, cytosol; 7, plasma membrane. The asterisk (*) indicates that in these samples only marginal amounts of the respective compound were detected. Error bars represent the standard deviation. ERG, ergosterol; LAN, lanosterol; SQ, squalene.

Chapter 3

# Influence of Squalene on Lipid Particle/Droplet and Membrane Organization in the Yeast *Saccharomyces cerevisiae*

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Running title: Squalene in lipid particles and membranes

Key words: squalene, ergosterol, lipid droplet, plasma membrane, fluidity, yeast.

Abbreviations: SQ: squalene; SE: steryl ester(s); TG: triacylglycerol(s); QM: quadruple mutant; LP, lipid particle; DPH, 1,6-diphenyl-1,3,5-hexatriene; TMA-DPH, 1(4-(trimethylamino)phenyl)-6-phenyl-1,3,5-hexatriene

## Abstract

In a previous study (Spanova *et al.*, 2010, J. Biol. Chem., 285, 6127-6133) we demonstrated that squalene, an intermediate of sterol biosynthesis, accumulates in yeast strains bearing a deletion of the *HEM1* gene. In such strains, the vast majority of squalene is stored in lipid particles/droplets together with triacylglycerols and steryl esters. In mutants lacking the ability to form lipid particles, however, substantial amounts of squalene accumulate in organelle membranes. In the present study, we investigated the effect of squalene on biophysical properties of lipid particles and membranes and compared these results to artificial membranes. Our experiments showed that squalene reduced the order of steryl ester shells in lipid particles. The majority of squalene, however, was localized to the center of lipid particles where it formed a soft core together with triacylglycerols. This view was confirmed with model lipid particles. In biological membranes it is more difficult to pinpoint the role of squalene because multiple effects such as levels of sterols and unsaturated fatty acids contribute to physical membrane properties. Fluorescence spectroscopic studies using endoplasmic reticulum, plasma membrane and artificial membranes revealed that it is not the absolute squalene level in membranes *per se*, but the squalene to ergosterol ratio which mainly affects membrane fluidity/rigidity. In a fluid membrane environment squalene induces rigidity of the membrane, whereas in rigid membrane there is almost no additive effect of squalene. In summary, our results demonstrate that squalene (i) can be well accommodated in yeast lipid particles and organelle membranes without causing deleterious effects; and (ii) although not being a typical membrane lipid may be regarded as a mild modulator of biophysical membrane properties.

# Introduction

The isoprenoid squalene is an important precursor for the biosynthesis of sterols, steroids and ubiquinons. Due to its beneficial properties as antioxidant [1] and dyslipidemic properties, and its possible use for prevention from cancer and skin disorders [2-4] the biotechnological production of squalene has been studied in several organisms [5-8]. Besides the occurrence of squalene in shark liver oil, olive oil, amaranth oil and some microorganism the yeast *Saccharomyces cerevisiae* was shown to accumulate squalene under certain culture conditions or/and by genetic modifications [9-14]. Under standard growth conditions squalene does not accumulate in the yeast cell but is converted to sterols. The ergosterol biosynthetic pathway is strictly aerobic and heme-dependent. Heme is necessary for the activity of the sterol-14-α-demethylase Erg11p, an NADPH-heme-dependent P450 protein [15;16]. If heme cannot be formed, e.g., in *hem1*Δ strains, squalene and lanosterol accumulate [13]. For studies described here we used strains deleted of *HEM1* which encodes the first enzyme in heme synthesis, δ-aminolevulinic acid synthase. Such *hem1*Δ strains have been widely used as a model for anaerobic growth [17;18] because metabolic changes resulting from the lack of cytochromes were similar to oxygen deficiency. As a consequence of such manipulations, anaerobic yeast and *hem1*Δ cells become auxotrophic for sterol and unsaturated fatty acids [19-22].

In the yeast as in other cell types lipid storage occurs in lipid droplets/particles. Steryl esters (SE) and triacylglycerols (TG) are the major storage lipids of *Saccharomyces cerevisiae* comprising roughly 50% of the lipid particles (LP) mass, each. Previous studies have shown that LP are highly flexible and dynamic organelles [23]. However, little is known about molecular processes involved in LP biogenesis. Investigations in several laboratories including our own favor a budding model [24-26]. This model is based on the hypothesis that SE and TG synthesized between the bilayer of endoplasmic reticulum (ER) form the core of nascent LP. After a certain particle size has been reached, the LP bud off the ER and becomes an independent organelle. Recently, we showed that only one of the SE and TG synthesizing enzyme of the yeast, Dga1p, Lro1p, Are1p or Are2p, is sufficient to form LP [27]. In the course of these studies a new structural model of LP was proposed suggesting that below the temperature of 18°C a random core consisting of TG is surrounded by SE shells and covered by a phospholipid (PL) monolayer with a small amount and number of proteins embedded [23;27]. Under standard growth conditions

only a small amount (0.5% of total mass) of squalene was found in LP [23;28]. In a *hem1Δ* strain accumulating squalene, approximately 70 % of its cellular amount are stored in LP [13]. This result led us to speculate that in such strains the physical properties of LP might be changed. To address this question we used differential scanning calorimetry as a method to identify order-disorder transitions of lipid components in LP [27]. As will be shown in this study, squalene has indeed an effect on the structural organization of LP.

Besides its localization in LP, squalene was also detected in organelle membranes of a *hem1Δ* strain [13]. This result is in line with previous findings showing squalene accumulation in membranes of yeast cells grown anaerobically [29]. These findings were surprising because squalene is a highly hydrophobic molecule which lacks the polar head group usually expected for membrane bilayer forming components such as phospholipids. It has been proposed that squalene or its saturated form squalane were accommodated horizontally in the middle of a lipid bilayer thus altering structure and properties of artificial membranes [30-32].

Our biological model of organelle membranes from a *hem1Δ* yeast mutant which accumulated squalene enabled us to test the possible impact of squalene on biophysical and biological properties of membranes. These studies were specifically performed with membranes from a *hem1Δ* mutant in a *dga1Δlro1Δare1Δare2Δ* (quadruple mutant, QM) background. This QM is devoid of all four acyltransferases, Dga1p, Lro1p, Are1p and Are2p which catalyze the synthesis of TG and SE in the yeast [33]. As a consequence of the quadruple deletion, such strains do not form LP. A QM*hem1Δ* mutant turned out as a valuable tool to accumulate squalene in subcellular membranes, especially in the endoplasmic reticulum and the plasma membrane. Since the QM*hem1Δ* mutant lacks LP but accumulates the same amount of squalene as *hem1Δ*, the squalene concentration in membranes was dramatically increased [13]. Using this experimental system we demonstrated that squalene in combination with ergosterol is an important parameter to modulate the fluidity of ER and plasma membrane. Our studies also took into account the multiple alterations of membrane components in *hem1Δ* deletions strains which additionally affect membrane properties.

## Experimental procedures

*Strains, culture conditions and subcellular fractionation*

Yeast strains used in this study are listed in Table 1. Inactivation of the *HEM1* gene was performed by transformation of the *hem1::URA3* disruption cassette according to Gueldener *et al.* [34] using folloving primers, HEM1 fw 5'-CTT CTC CAT TCC GTC AGC TGA TAC TCT ATT CGG TTG TGT GTT GCA CAG CTG AAG CTT CGT ACG C-3' and HEM1rev 5'-ATA CTC ATA CGT TTC TCT CTC TTT ACT TTC TGT ACC CCC GAG GGC ATA GGC CAC TAG TGG ATC TG-3'. The disruption of *HEM1* in mutants was verified by standard colony PCR and sequencing.

Cells were grown aerobically to the early stationary phase at 30°C in YPD medium containing 1% yeast extract (Oxoid), 2% peptone (Oxoid) and 2% glucose (Merck). Media were inoculated with pre-cultures to the $A_{600}$ of 0.1. Heme deletion strains were grown on YPD medium supplemented with 20 μg/ml ergosterol (Erg) and 0.06% Tween 80 as a source of oleic acid.

The yeast lipid particle (LP) fraction was obtained at high purity from cells grown to the stationary phase as described by Leber *et al.* [23]. Isolation of microsomes (ER) and plasma membrane used in this study was described by Zinser *et al.* [35]. The quality of subcellular fractions was routinely tested by Western blot analysis (see below).

*Protein analysis*

Proteins were quantified by the method of Lowry *et al.* [36] using bovine serum albumin as a standard. Polypeptides were precipitated with trichloroacetic acid and solubilized in 0.1% sodium dodecyl sulfate (SDS), 0.1 M NaOH prior to quantification. Samples of the LP fraction were delipidated prior to protein quantification. Non-polar lipids were extracted with 2 volumes of diethyl ether, the organic phase was withdrawn, residual diethyl ether was removed under a stream of nitrogen, and proteins were precipitated as described above.

SDS-polyacrylamide gel electrophoresis was carried out by the method of Laemmli [37]. Samples were denatured at 37°C to avoid hydrolysis of polypeptides. Proteins on gels were detected by staining with Coommassie Blue. Western blot analysis was performed as described by Haid and Suissa [38]. Proteins were detected by enzyme-linked immunosorbent assay (ELISA)

using rabbit antiserum as primary antibody and peroxidase-conjugated goat anti-rabbit IgG as second antibody. Primary antibodies used in this study were from rabbits and directed against yeast Ayr1p, Por1p, Wbp1p and Gas1p. The enhanced chemiluminescent signal detection kit SuperSignal™ (Pierce Chemical Company, Rockford, IL, USA) was used to visualize immunoreactive bands and then quantified densitometrically.

*Lipid analysis*

Lipids from yeast cells were extracted as described by Folch *et al.* [39]. Total phospholipids were quantified from lipid extracts by the method of Broekhuyse [40]. For quantification of neutral lipids, lipid extracts were applied to Silica Gel 60 plates, and chromatograms were developed in an ascending manner by a two step developing system [41]. First, chromatograms were developed using light petroleum:diethyl ether:acetic acid (70:30:2; per vol.), and then light petroleum:diethyl ether (49:1; v/v) as solvents. To visualize separated bands, TLC plates were dipped into a charring solution consisting of 0.63 g $MnCl_2$ x $4H_2O$, 60 ml water, 60 ml methanol and 4 ml concentrated sulphuric acid, briefly dried and heated at 100°C for 20 min. Then, lipids were quantified by densitometric scanning at 400-650 nm with squalene, triolein and cholesteryl ester as standards using a Shimadzu dual-wave length chromatoscanner CS-930.

Squalene and individual sterols (free sterols and SE) from whole cells or subcellular fractions were identified and quantified by gas liquid chromatography – mass spectrometry (GLC-MS) [42]. GLC-MS was performed on an HP 5890 Gas-Chromatograph equipped with a mass selective detector HP 5972, using an HP5-MS capillary column (30 m x 0.25 mm, 0.25 μm film thickness). Aliquots of 1 μl were injected in the splitless mode at 270°C injection temperature with helium as carrier gas at a flow rate of 0.9 ml/min in constant flow mode. The following temperature program was used: 1 min at 100°C, 10°C/min to 250°C, and 3°C/min to 310°C. Mass spectra were acquired in the scan mode (scan range 200-550 amu) with 3.27 scans per second. Sterols were identified based on their mass fragmentation pattern.

Fatty acids were analyzed by GLC. Lipid extracts prepared as described above were incubated with hot 2.5% sulphuric acid in methanol. After incubation, water was added, and the converted methyl esters were extracted with light petroleum. Fatty acid methyl esters were separated using a Hewlett-Packard 6890-Gas-Chromatograph equipped with a HP-INNO Wax

capillary column (15 m × 0.25 mm i.d. × 0.50 μm film thicknesses) and helium as carrier gas. Fatty acids were identified by comparison to commercial fatty acid methyl ester standards (NuCheck, Inc., Elysian, MN, USA).

*Preparation of phospholipid vesicles*

Lipid extracts prepared as described above were taken to dryness and dispersed in 10 mM Tris/Cl, pH 7.4. Vesicles were prepared by shaking the suspension for 24 hours at 30°C. For the preparation of squalene containing vesicles, squalene was added to lipid extracts, and vesicles were prepared as described above.

*Fluorescence anisotropy*

Isolated microsomes and plasma membrane were resuspended in 10 mM Tris/Cl, pH 7.4 at the same phospholipid concentration. After addition of diphenylhexatriene (DPH) or trimethylammonium-diphenylhexatriene (TMA-DPH) at a molar ratio of 1:50 probe to phospholipid, mixtures were incubated for 5 min at 30°C. Samples were kept in the dark as long as possible. Then, fluorescence measurements were carried out using a Shimadzu RF 540 spectrofluorimeter equipped with polarizers in the excitation and emission light path. Excitation and emission wavelengths for DPH were 350 and 452 nm (slit width 10 nm), and for TMA-DPH 355 and 430nm (slit width 10 nm). Fluorescence intensities were corrected for background fluorescence and light scattering from the unlabelled sample. The fluorescence anisotropy r was calculated according to the equation

$$r = (I_{\parallel} - I_{\perp}) / (I_{\parallel} + 2.I_{\perp})$$

$I_{\parallel}$ and $I_{\perp}$ are measured emission intensities parallel and perpendicular to the vertical polarization plane of the excitation light [43].

*Differential scanning calorimetry (DSC)*

Differential scanning calorimetry experiments were performed as described previously [27] using a VP-DSC instrument from MicroCal, Inc. Northampton, MA, USA. Samples of LP prepared from wild type and mutant strains were used undiluted. A buffer containing 0.25 M sorbitol, 10 mM MES/Tris and 0.2 mM $Na_2EDTA$ (pH 6.9) was used to fill the reference cell. Heating scans were performed at a scan rate of 15°C/h between 1°C and 65°C. Pre-scan

thermostating at 1°C was allowed for 60 min. Enthalpies were calculated by integrating the peak area after baseline adjustment and normalization to SE concentrations using the MicroCal Origin software (VP-DSC version).

To mimic the conditions in *hem1Δ* LP, "squalene enriched" LP were prepared by addition of squalene to wild type LP. For this purpose, LP of wild type were added to a squalene film of appropriate concentration dried under a stream of nitrogen to reach the squalene to SE ratio of LP from a *hem1Δ* strain. Artificial LP were incubated at 30°C and vortexed several times to integrate squalene. Measurement and analysis of data were performed as described above.

# Results

*Squalene influences the structure of yeast lipid particles*

In a recent study from out laboratory we proposed a new model of LP structure based on results obtained by differential scanning calorimetry (DSC) and small angle X-ray scattering [27]. It was shown that below the phase transition temperature of 18°C SE of LP form shell like layers which surround a core of TG. In another recent investigation we showed that squalene can become a prominent LP component when accumulated in a *hem1Δ* yeast strain [13]. We speculated that the presence of squalene in LP may affect the order and the structural organization of the droplet. To test this hypothesis we performed differential scanning calorimetry with LP variants isolated from a *hem1Δ* mutant. Results of these measurements are displayed in Figure 1. As described previously [27] all significant transitions seen in these thermographs are attributed to melting of the acyl chains of SE, since amounts of other LP components such as proteins or phospholipids are negligible. As negative controls we used LP from *are1Δare2Δdga1Δ*, *are1Δare2Δlro1Δ* and *are1Δare2Δhem1Δ* strains [27] which cannot produce SE. In all LP variants lacking SE no transition signals were detected (data not shown). The thermogram of LP from wild type exhibited two distinct peaks named S1 (melting point 35°C) and S2 (melting point 17°C) (Figure 1 top). This finding is similar to our previous results which showed one major peak at 18°C and a smaller one above 30°C [27]. In the present study, data were normalized to the amount of protein which enabled us to compare the different types of LP. Furthermore, we used more concentrated samples which allowed a more distinct detection of changes induced by temperature shifts. The two peaks in the thermograms represent two different types of ordered SE shells present in the LP. One type of SE layers (S2) which is less ordered and probably contains a higher amount of unsaturated FA melts at lower temperature (17°C). We assume that this portion of SE layers is close to the center of the LP and disturbed by the presence or infiltration of TG. The other type of SE layers (S1) is more ordered, melts above 30°C and seems to be located at the LP periphery. Probably, layers of S1 also contain more saturated FA. S1 layers are far away from the LP core and not disturbed by TG, but may be influenced by proteins present in the LP surface membrane. The rigidity of the S1 layer may support the phospholipid surface monolayer and help to maintain the stability of LP.

Differences between the thermographs of LP from wild type and *hem1Δ* are displayed in Figure 1 (top and bottom, respectively). Again, we observed two separate peaks derived from two

different type of SE shells, but transition temperatures decreased to 27.2°C (S1) and 7.5°C (S2), respectively, and the enthalpy of the transitions was markedly reduced (Table 2). This result indicates that squalene present in *hem1Δ* LP decreases the amount of ordered SE shells. These data, however, have to be interpreted with caution, since additional differences in the composition of the two LP variants have to be taken into account. As can be seen from Table 3, the amount of squalene was dramatically increased in LP from *hem1Δ*, mostly at the expense of SE, compared to wild type. Moreover, the degree of fatty acid saturation was much higher in the mutant than in wild type. Considering that signals in the thermogram are derived only from SE, we can interpret our results as follows. The decreased signal intensity of S1 and S2 peaks may be attributed to lower amounts of SE in *hem1Δ*, but also to some randomization caused by interaction of SE with TG and squalene. The altered lipid composition of LP from *hem1Δ* may cause drifting of a substantial portion of SE to the LP core consisting of TG and squalene. However, decreased transition temperatures of S1 and S2 in LP from *hem1Δ* clearly indicated some loss of order in the existing SE shells.

Since we could not unambiguously attribute effects described above to squalene which accumulated in *hem1Δ* LP we performed as control experiments DSC using wild type LP non-supplemented (Figure 1, top) or supplemented with squalene (Figure 1, middle). The latter samples had the same SE to squalene ratio as LP from *hem1Δ* and showed similar decreased enthalpy of SE transitions and shift of the signals to lower temperatures, although at a lesser extent. These results clearly confirmed the specific effect of squalene on the LP structure. The observed effect was at the same time a proof for the successful incorporation of squalene into LP, because the composition of the two LP variants was otherwise completely the same. As a further control, we performed DSC experiments with “artificial LP” created by mixing phospholipids, TG and SE with or without squalene. As in experiments with LP from *hem1Δ* and wild type LP supplemented with squalene, we found a shift of peaks to lower temperatures and a decrease of peak intensity when squalene was present (data not shown). These experiments also excluded possible effects of proteins on DSC measurements, which was not surprising because polypeptides are present in LP only at minor amounts.

*Squalene affects yeast organelle membranes*

Our previous studies had shown that in the *dga1Δlro1Δare1Δare2Δhem1Δ* (QM*hem1Δ*) which is unable to produce TG and SE and does not contain LP, squalene formed at large amounts due to the deletion of *HEM1* is stored in the ER and plasma membrane at substantial concentrations [13]. When we performed growth tests with wild type, *hem1Δ,* QM and QM*hem1Δ* we realized that strains accumulating squalene exhibited growth defects at pH 4.0, pH 8.0, in the presence of 0.7 M NaCl and in the presence of 2 % DMSO (Figure 2). We took these results as a hint that membranes of *hem1Δ* strains, especially the plasma membrane may suffer from the presence of squalene. These observations led us to study properties of the plasma membrane and the endoplasmic reticulum in *hem1Δ* and QM*hem1Δ* in detail. For this purpose, microsomes and plasma membrane from the respective strains and from the corresponding controls were isolated. Western Blot analysis using markers for plasma membrane (Gas1p), microsomes (Wbp1p), lipid particles (Ayr1p) and mitochondria (Por1p) revealed that all preparations were obtained at high quality and purity (Table 3) in agreement with previously published results [35].

To test membrane fluidity/rigidity biological membranes we employed steady-state fluorescent anisotropy. Fluorescent probes used to assess the physical state of the membrane samples were 1,6-diphenyl-1,3,5-hexatriene (DPH), a non-polar compound probably inserting into membranes with a less defined orientation within the hydrophobic region of the bilayer [44]; and its cationic derivate 1(4-(trimethylamino)phenyl)-6-phenyl-1,3,5-hexatriene (TMA-DPH), whose positively charged quaternary amino group is anchored at the membrane surface and interacts with water thus leading to a well characterized label orientation perpendicular to the membrane normal [45].

In contrast to artificial membranes consisting of defined multi-component system, data obtained with biological membranes are much more difficult to explain and need to be interpreted with caution. The fluidity of a membrane depends on various components such as the amount of ergosterol, the degree of fatty acid saturation and the concentration of polypeptides present in a lipid bilayer. Since ER and plasma membrane samples used for anisotropy measurements were derived from four different strains, namely wild type, *hem1Δ*, QM and QM*hem1Δ*, we analyzed the respective samples for the above mentioned parameters (Figures 3 and 4). These data were included in the interpretation of anisotropy measurements.

In microsomes (ER), the anisotropy measured with DPH and TMA-DPH was significantly higher in samples derived from *hem1Δ* and QM*hem1Δ* mutants than in the corresponding control strains (Figure 3). These values indicated that mutant membranes were more rigid than wild type. As mentioned above, this result represents an overall effect which is caused by various parameters. As can be seen from Figure 3, the ratio ergosterol to phospholipids decreased in *hem1Δ* and QM*hem1Δ*, but the degree of fatty acid saturation, the protein to phospholipid ratio and the squalene to phospholipid ratio was increased. It is noteworthy that ergosterol and unsaturated fatty acids derived from external supplementation. Whereas the decrease of the ergosterol content in microsomes from *hem1Δ* would increase membrane fluidity, enhanced amount of proteins and saturated fatty acids present in the membrane cause the opposite effect. Thus, the ergosterol as a possible reason for higher rigidity in *hem1Δ* microsomes was excluded. However, the question as to the effects of proteins and saturated fatty acids remained. To clarify this point we performed anisotropy measurements with artificial membrane vesicles consisting of lipids extracted from microsomes of wild type, *hem1Δ*, QM and QM*hem1Δ* (Table 5). To these samples, squalene was added at an amount corresponding to the squalene concentration in *hem1Δ* samples. The double amount of squalene was used to test for additional effects on membrane fluidity. As can be seen from Table 5 the anisotropy measured with DPH increased significantly after addition of squalene to wild type membranes, but further addition of squalene had no additive effect. The same observation was made with TMA-DPH. In lipid extracts from *hem1Δ* microsomes, addition of squalene had no effect with DPH as a probe. We assumed that squalene already present in this extract had led to a basis level of rigidity in the membrane which was not further increased. The increase of anisotropy in *hem1Δ* upon addition of squalene measured with TMA-DPH may be attributed to an additive effect. It has to be considered that DPH is a probe for entirely hydrophobic membrane regions, whereas TMA-DPH also acts at least in part as a probe of the membrane interface. In the QM the situation was different. The anisotropy value was already as high as in *hem1Δ* without addition of squalene and significantly higher than in wild type. Addition of squalene had no effect when measured with DPH, but caused some increase of the anisotropy with TMA-DPH. In QM*hem1Δ* addition of squalene was without effect with both probes. In summary, these results indicate that incorporation of squalene into microsomal membranes increased rigidity. If membranes were already rigid, e.g., by the presence of a basis level of squalene, further addition of this isoprenoid led to an attenuation.

Anisotropy measurements with plasma membrane samples using DPH or TMA-DPH as probes revealed that in wild type values were already higher than with microsomes (see Figure 4). This effect is result of the high ergosterol to phospholipid ratio and the large amount of unsaturated fatty acids present in the plasma membrane. In plasma membrane preparations from *hem1Δ* and QM*hem1Δ*, respectively, the anisotropy was not significantly increased over controls. Again, the specific effect of squalene in these samples was difficult to explain, and experiments with lipid extracts from different samples were performed. In lipid vesicles derived from of wild type and *hem1Δ* plasma membrane addition of squalene did not increase the anisotropy with DPH, and only slightly with TMA-DPH. We conclude from our results that in these samples the squalene effect was only minor compared to the more dominant effects of other membrane components. In vesicles from QM and QM*hem1Δ* plasma membrane, the basic anisotropy was significantly lower than in wild type (see Table 5). This effect was attributed to the markedly decreased ergosterol to phospholipid ratio and decreased amount of unsaturated fatty acids (see Figure 4) in QM and QM*hem1Δ*. In QM samples, the addition of squalene had an increasing effect on anisotropy, but in vesicles from QM *hem1Δ* plasma membrane which contains some squalene the effect was only observed with TMA-DPH.

In summary, squalene appears to increase rigidity in membranes which are rather fluid such as microsomes. In membranes with a high level of basis rigidity the influence of squalene is minor to negligible. Finally, it has to be noted that effects observed with TMA-DPH are more pronounced that with DPH. This result suggested that squalene rather caused interfacial than internal membrane alteration.

## Discussion

Storage of non-polar lipids in all types of cells including yeast occurs in a compartment named lipid particle (LP)or lipid droplet [46]. In previous days, LP were regarded as an inert subcellular structure. This view has changed insofar as we know by now that certain enzymes of LP may play an important metabolic role [47]. More recently, we were also able to demonstrate an internal structure of LP [27]. These studies were extended by investigations presented here. The structure of LP from *Saccharomyces cerevisiae* wild type cells is rather simple. A core consisting of TG is surrounded by SE shells with increasing order from the interior of the LP to the periphery (Figure 5A). The surface of LP is covered by a phospholipid monolayer with a small amount of proteins embedded. While less rigid SE shells (S2) melt around 17°C, more rigid S1 layers melt close above 35°C. It appears that higher ordered S1 shells localize close to the surface phospholipid monolayer and help to stabilize the LP.

In previous studies with the yeast *Saccharomyces cerevisiae* [13], we showed that squalene accumulated in *hem1Δ* strains and localized mainly to LP. Squalene is highly hydrophobic and expected to mix with other non-polar lipids of the LP such as TG and SE. Differential scanning calorimetry performed with such LP variants showed that SE layers of type S1 and S2 are disturbed and lose some order due to the presence of squalene (Figure 5B). Most likely, a marked portion of SE together with squalene and TG is localized to the soft core of LP. Alternatively or in addition, a smaller portion of squalene may intercalate into SE layers and decrease their order. In summary, our experiments indicate that LP containing squalene are softer than those without squalene. This effect may lead to lower stability of the droplet and hence be important for the access of hydrolytic enzymes such as TG lipases to the storage components of LP. This view is in agreement with the result that S1 layers in LP of the *hem1Δ* strain melt at lower temperature and confer better access to stored sterols, which are rare under these conditions and thus important for the viability of the mutant strain.

Our previous studies [13] also had shown that due to genetic manipulations squalene can be accommodated at substantial amount in yeast subcellular membranes. We hypothesized that the presence of squalene in membranes, especially in the plasma membrane, may affect growth of the yeast and/or cause sensitivity to external stress. Hauss *et al*. [31] had advocated such a model using squalane in artificial membranes. These authors showed, however, that proton flux was

affected by the presence of squalane in the membrane bilayer. Growth studies presented here (see Figure 2) showed that *hem1Δ* mutants become sensitive to low and high pH, high salt concentration and to dimethylsulfoxide. In biological membranes, however, this effect cannot be attributed to a single component, and alteration of the membrane composition from different mutants had to be included in the interpretation.

Also anisotropy measurements with ER and plasma membrane are result of overall effects caused by different components. To pinpoint the squalene influence in detail, we performed in parallel studies with artificial membranes. It appears that in soft membranes squalene has some stabilizing effect making the membranes more rigid. These observations are in line with experiments using model system membranes [30-32]. In such model membranes, squalene is most likely not localized axially in the membrane, but in the interior of the bilayer.

Interestingly, anisotropy effects observed with microsomal membranes and plasma membrane, and with lipid extracts from both samples differed. In soft microsomal membranes, squalene had a clear stabilizing effect, whereas in the more rigid plasma membrane the influence of squalene was less pronounced. We conclude that the ratio of ergosterol to squalene is crucial for a possible stabilizing effect of squalene. Surprisingly, no effect of the squalene in artificial membranes was observed by Simon *et al.* [32] when they studied interaction of squalene with liposomes and monolayers of dipalmitoyl phosphatidylcholine. These authors used large amounts of squalene (10% - 50% mol) and thus studied most likely monolayer covered droplets instead of bilayers with a thick layer of squalene in between. In our experiments, we observed minor changes, since we used only squalene concentrations up to 0.4 mol%. On the other hand, Lohner *et al.* [30] showed that squalene at a concentration of 6 mol % in pure phospholipid vesicles altered the lamellar-to-inverse-hexagonal phase transition with increasing the size of inverse hexagonal phase tubes. These authors argued that squalene must be stored in a most disordered region of the bilayer, evoking that squalene rather exists in a coiled than extended conformation and localizes to the interior of the bilayer. Our experiments using biological and model membranes extend this model insofar as squalene in the ER may rather adapt to a conformation close to ergosterol, whereas in the plasma membrane squalene might prefer the coiled conformation. In any case, the membrane modulating role of squalene is a novel facet of biophysical properties of this lipid.

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# Tables

Table 1. **Yeast strains used in this study**

| Yeast strains | Genotype | Source or Ref. |
|---|---|---|
| Wild type W303 | *SUC2 GAL+ mal mel ade2-1 can1-100 his3-11,15 leu2-3 112 trp1-1 ura3-1* | ScanBi Ltd., Alnarp, Sweden [33] |
| QM *dga1Δ lro1Δ are1Δ are2Δ* | *W303; MATα dga1::KanMX4 lro1::TRP1are1::HIS3 are2::LEU2 ADE2 met ura3* | ScanBi Ltd., Alnarp, Sweden [33] |
| *hem1Δ* | *W303; MATa hem1::URA3 his3 leu2 trp1* | This study |
| QM*hem1Δ* | *W303; MATα dga1::KanMX4 lro1::TRP1are1::HIS3 are2::LEU2 hem1::URA3 ADE2 met* | This study |

Table 2. **Thermodynamic parameters of lipid particles from *S. cerevisiae* wild type W303 without and with addition of squalene and mutant *hem1Δ***

$T_{mS2}$: temperature transition of lower ordered SE; $T_{mS1}$: temperature transition of higher ordered SE; $\Delta H_{cal,S2}$: enthalpy of low temperature transition; $\Delta H_{cal,S1}$: enthalpy of high temperature transition. SQ, squalene. Enthalpy is given per mole SE per mg protein.

| | $T_{mS2}$ | $\Delta H_{cal,S2}$ | $T_{mS1}$ | $\Delta H_{cal,S1}$ |
|---|---|---|---|---|
| | *°C* | *kcal/(°C mole mg)* | *°C* | *kcal/(°C mole mg)* |
| **W303** | 17.3 | 1.47 | 35.0 | 0.40 |
| **W303 + SQ** | 14.6 | 0.79 | 33.7 | 0.28 |
| ***hem1Δ*** | 7.4 | 0.60 | 27.3 | 0.21 |

Table 3. **Lipid composition wild type and *hem1Δ* lipid particles**

Lipid particles were isolated and analyzed as described under *Experimental Procedures*.
SQ, squalene; SE, steryl esters; SFA, saturated fatty acids; UFA, unsaturated fatty acids

| | | % of total mass in LP | UFA/SFA |
|---|---|---|---|
| **W303** | SQ | 1.28 ± 0.37 | |
| | SE | 63.24 ± 3.66 | 4.28 ± 1.35 |
| | TG | 35.48 ± 3.29 | 9.26 ± 4.47 |
| ***hem1Δ*** | SQ | 23.81 ± 2.78 | |
| | SE | 6.80 ± 1.92 | 2.22 ± 0.16 |
| | TG | 69.41 ± 0.86 | 0.76 ± 0.48 |

Table 4. **Quality control of yeast subcellular fractions**

Subcellular fractions were isolated as described under *Experimental Procedures*. The relative enrichment of markers in the homogenate was set as 1. Marker proteins: Gas1p, β-1,3-glucanosyltransferase (marker for plasma membrane); Wbp1p, β-subunit of the oligosaccharyl transferase glycoprotein complex (marker for microsomes); Por1p, porin (marker for mitochondria); Ayr1p, acyldihyroxyacetonephosphate acyltransferase (marker for lipid particles). n.d., not detectable; -, not measured

Relative enrichment (fold)

| | **wild type** | | | | ***hem1Δ*** | | | | **QM** | | | **QM*hem1Δ*** | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | Gas1p | Wbp1p | Por1p | Ayr1p | Gas1p | Wbp1p | Por1p | Ayr1p | Gas1p | Wbp1p | Por1p | Gas1p | Wbp1p | Por1p |
| Microsomes 30.000 *g* | n.d. | 2.3 ± 0.3 | 0.35±0.11 | n.d. | n.d. | 5.4 ± 0.9 | 0.1 ± 0.07 | n.d. | n.d. | 3.5 ± 0.1 | n.d. | n.d. | 1.7 ± 0.2 | n.d. |
| Plasma membrane | ~100 | 0.02 ± 0.03 | 0.22±0.09 | n.d. | ~100 | 0.2 ± 0.2 | 1.3 ± 0.2 | n.d. | ~100 | 1.8 ± 0.2 | 1.1 ± 0.2 | ~100 | 0.32 ± 0.19 | 0.15 ± 0.03 |
| Mitochondria | - | - | 2.9 ± 0.2 | - | - | - | 2.9 ± 0.2 | - | - | - | 2.9 ± 0.2 | - | - | 2.9 ± 0.2 |
| Lipid particles | - | - | - | ~100 | - | - | - | ~100 | | | | | | |

Table 5. **Anisotropy of artificial membrane vesicles from lipid extracts of isolated membranes.**

Organelles were isolated as described in Figure 2 and Figure 3. Vesicles from lipid extracts with or without addition of squalene addition were formed, and anisotropy was measured as described in Experimental Procedures.

+SQ, amount of squalene as in *hem1Δ*; +SQ+SQ, double amount of squalene as in *hem1Δ*; DPH, diphenylhexatriene; TMA-DPH, trimethylammonium-diphenylhexatriene; PM, plasma membrane

| Organelle | Source +additive | Anisotropy | |
|---|---|---|---|
| | | DPH | TMA-DPH |
| Microsomes | **W303** | 0.108 ± 0.005 | 0.244 ± 0.026 |
| | +SQ | 0.124 ± 0.018 | 0.262 ± 0.020 |
| | +SQ+SQ | 0.127 ± 0.018 | 0.246 ± 0.006 |
| | ***hem1Δ*** | 0.145 ± 0.005 | 0.221 ± 0.024 |
| | +SQ | 0.148 ± 0.009 | 0.254 ± 0.002 |
| | **QM** | 0.146 ± 0.003 | 0.200 ± 0.033 |
| | +SQ | 0.147 ± 0.005 | 0.261 ± 0.019 |
| | +SQ+SQ | 0.143 ± 0.002 | 0.250 ± 0.001 |
| | **QM*hem1Δ*** | 0.130 ± 0.006 | 0.235 ± 0.015 |
| | +SQ | 0.128 ± 0.032 | 0.222 ± 0.001 |
| PM | **W303** | 0.289 ± 0.005 | 0.308 ± 0.009 |
| | +SQ | 0.264 ± 0.006 | 0.314 ± 0.009 |
| | +SQ+SQ | 0.265 ± 0.001 | 0.297 ± 0.005 |
| | ***hem1Δ*** | 0.253 ± 0.055 | 0.280 ± 0.008 |
| | +SQ | 0.250 ± 0.025 | 0.300 ± 0.002 |
| | **QM** | 0.160 ± 0.006 | 0.217 ± 0.003 |
| | +SQ | 0.193 ± 0.009 | 0.304 ± 0.006 |
| | +SQ+SQ | 0.181 ± 0.005 | 0.273 ± 0.020 |
| | **QM*hem1Δ*** | 0.222 ± 0.012 | 0.236 ± 0.008 |
| | +SQ | 0.222 ± 0.001 | 0.271 ± 0.008 |

## Figures

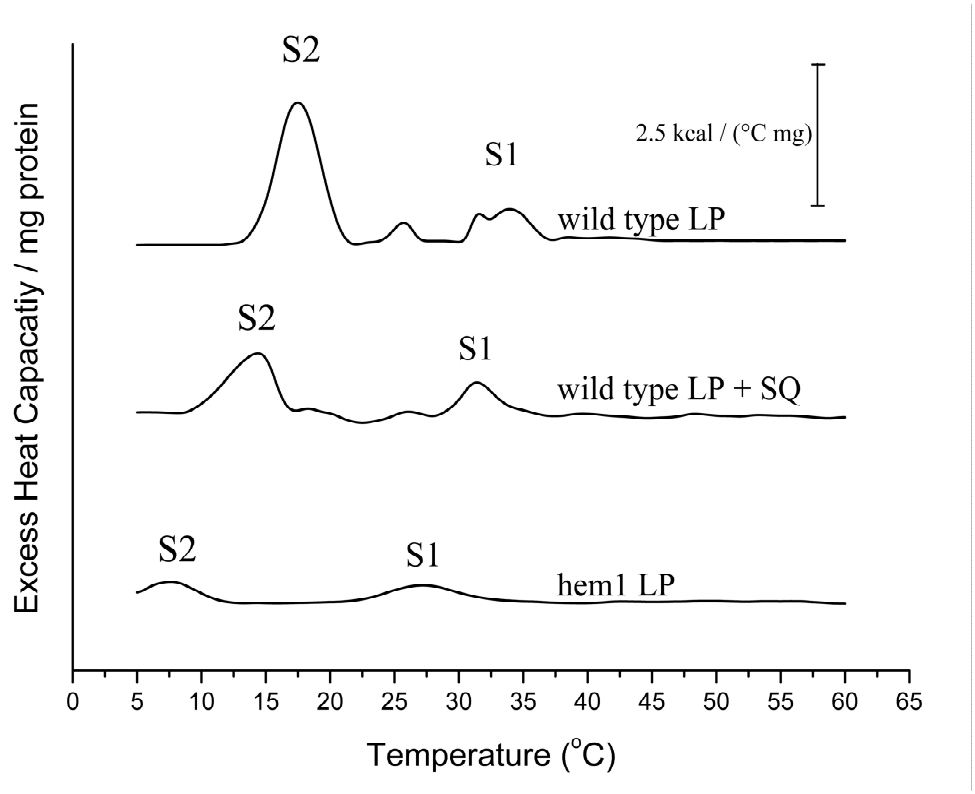


Figure 1: **Differential scanning calorimetry thermogram of lipid particles**

Heating scans of LP preparations from wild type W303 in the absence (top) or presence of squalene (middle), and from a *hem1Δ* mutant (bottom) are compared. The thermograms showing the second heating scans after one heating-cooling cycle (up to 25°C) are displaced on the y-axis by arbitrary units for the sake of clarity. The excess heat capacity was normalized to the amount of protein of the respective samples. The scan rate was 15°C/h. The analyzed data listed in Table 2 have been normalized to mole SE and mg protein.

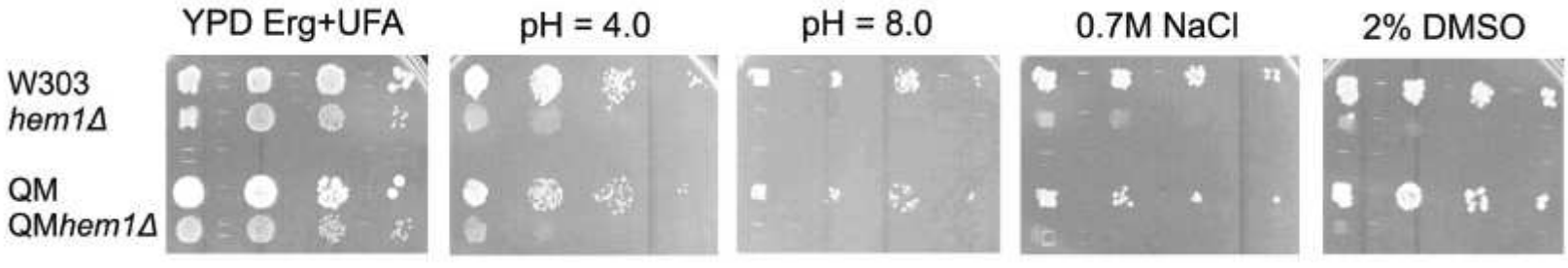


Figure 2: **Stress sensitivity of *hem1Δ* mutants**

Cells were grown on YPD plates supplemented with ergosterol and unsaturated fatty acids either at pH 4.0, in the presence of 0.7 M NaCl, or in the presence of 2% dimethylsulfoxide for 72 hours. Growth at pH 8.0 was monitored after 1week. YPD Erg+UFA, YPD medium with ergosterol and unsaturated fatty acids; DMSO, dimethylsulfoxide

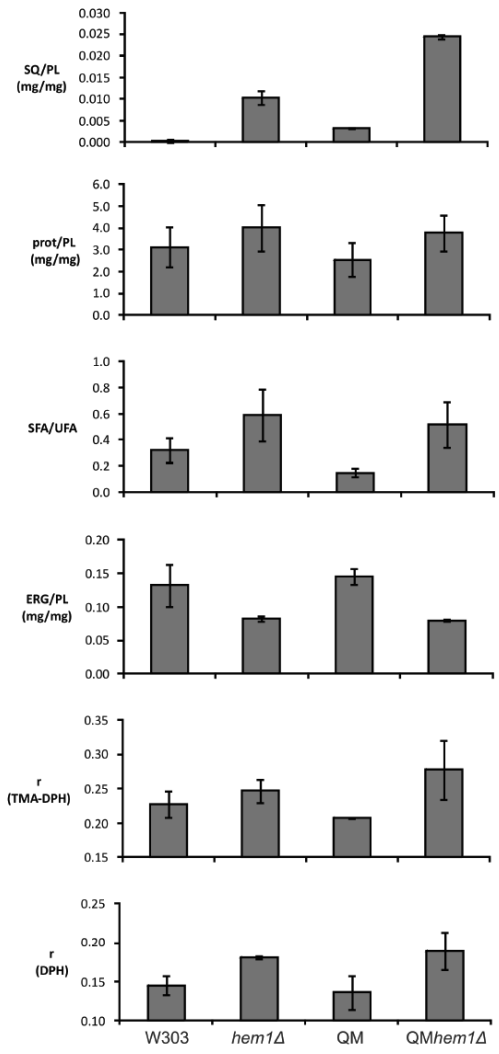


Figure 3: **Analysis of isolated microsomal membranes**

Cells were cultivated to the stationary phase with/without supplements, and microsomes were isolated as described by Zinser *et al.* [35]. Proteins were estimated by the method ofLowry *et al.* [36], total phospholipids were quantified by the method of Broekhuyse [40], and sterols were quantified by GLC-MS from aliquots containing a defined amount of protein. Anisotropy was measured as described in Experimental procedures. The asterisk (*) indicates that the value of standard deviation was less than 0.0005. SQ, squalene; PL, phospholipids; prot, proteins; SFA, saturated fatty acids; UFA, unsaturated fatty acids; ERG, ergosterol, DPH, diphenylhexatriene; TMA-DPH, trimethylammonium-diphenylhexatriene; r; anisotropy

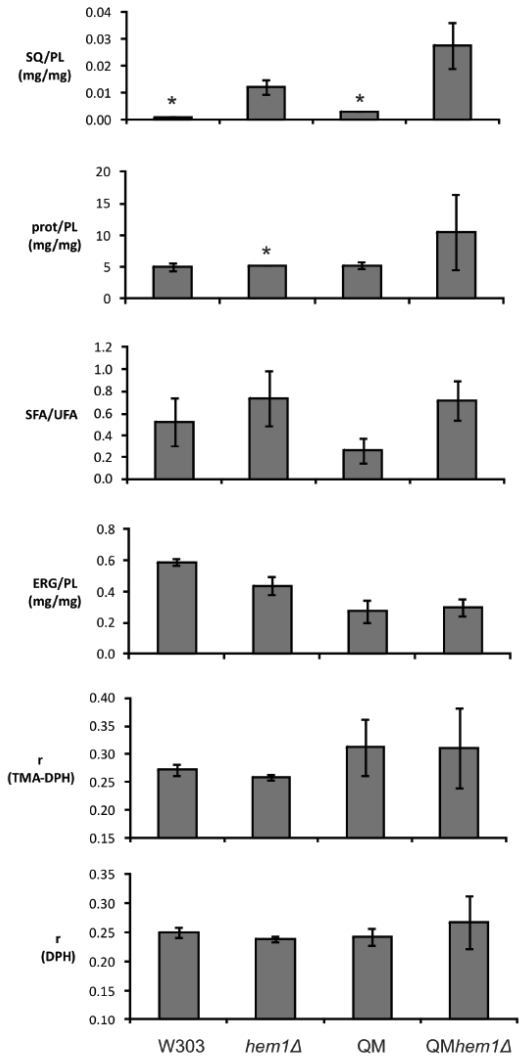


Figure 4: **Analysis of isolated plasma membranes**

Cells were cultivated to stationary phase with/without supplements, and plasma membrane was isolated as described by Zinser *et al.* [35]. For abbreviations see legend to Figure 3.

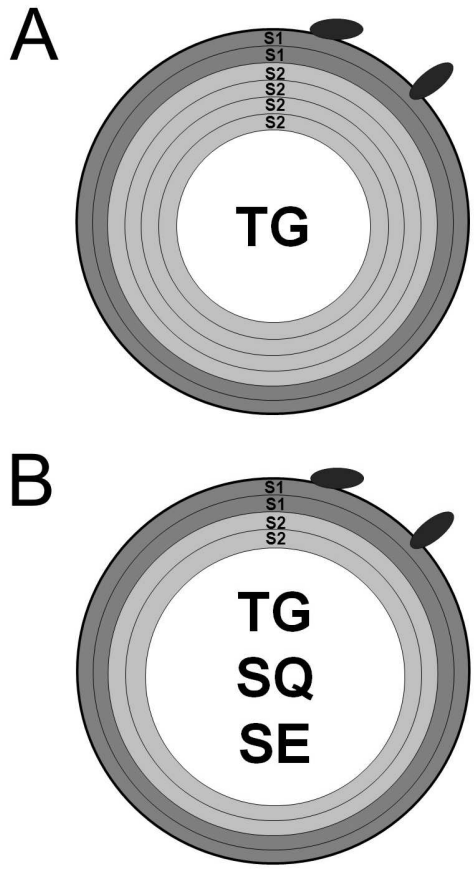


Figure 5: **Structural model of lipid particles influenced by squalene**

Model of lipid particles without squalene (A) or containing squalene (B). S1, densely packed SE shell; S2, randomized SE shell; SE, steryl ester; TG, triacylglycerol; SQ, squalene

**Summary and General Discussion**

The central aim of my Thesis was to study localization of squalene in yeast cells and its influence on lipid storage and membrane properties. These aspects have not been investigated before in great detail but turned out to be highly relevant for possible lipotoxic effects of this component. In brief, the following aspects were studied in this Thesis: (i) First, I investigated localization of squalene under accumulating conditions in the various subcellular compartments of the yeast *Saccharomyces cerevisiae*; (ii) secondly, I addressed squalene storage in lipid particles (LP) in some detail, and investigated subcellular distribution of squalene in yeast cells lacking neutral lipid synthesizing enzymes and thus lipid particles; (iii) finally, the influence of squalene on the LP structure and membrane properties was elucidated.

For this Thesis it was important to create situations causing accumulation of squalene in the cell thus provoking possible lipotoxicity. Therefore, I decided to study cells with deletion of the *HEM1* gene in wild type and *dga1Δlro1Δare1Δare2Δ* (quadruple mutant, QM) background to shed more light on the squalene distribution under conditions with enhanced cellular squalene level. It has to be mentioned that cells grown aerobically bearing a deletion of *HEM1* or anaerobically grown cell require supplementation with unsaturated fatty acids (presented as Tween80) and ergosterol, because both biosynthetic pathways require oxygen or the heme-dependent presence of cytochromes, respectively [1;2].

As mentioned above, the first hypothesis for this study was that squalene might predominantly accumulate in LP. This hypothesis turned out to be true. We showed, however, that squalene disturbed SE shells in LP and decreases the phase transition temperature. We concluded from these results that squalene made the LP particle as a whole softer either by recruiting SE from the surrounding shells to the core of the particle and/or by protruding into the existing SE shells. Details about the orientation of squalene in LP have still to be elucidated. We can only speculate at present that the form of squalene, either coiled or stretched, may influence its assembly in the LP.

In a *hem1Δ* strain over 70% of cellular squalene formed was stored in LP together with TG and SE. Under these conditions squalene was not lipotoxic which can be explained by the combined package with TG and SE and sequestering from the rest of the cell. It came as a surprise, however, that a *dga1Δlro1Δare1Δare2Δhem1Δ* (QM *hem1Δ*) mutant which lacks LP but produces large amounts of squalene was viable and did not form any LP like structures. In

this strain squalene was found in all cellular membranes at reasonably high amounts. Thus, it appears that the biomembrane system of the yeast is highly flexible and can buffer quite well potentially disturbing hydrophobic components.

The question remains how squalene finds its way to its proper destinations. It appears that squalene synthesized in the ER forms nascent LP together with TG and SE synthesized also in the ER. The combination of components obviously allows initiation of the LP budding process which does not occur when squalene is the only non-polar lipid formed in the ER. Squalene alone does not initiate LP formation but is distributed to membranes. This finding suggests a mechanism of squalene transport in the cell. In principle, such a mechanism may not only be restricted to the supply of squalene to membranes but also to LP. The possibility of combined mechanisms should also be taken into account. Squalene transport to LP and membranes is currently only a matter of speculation because several pathways are possible. Squalene may be transported by the same mechanisms as sterols, namely either by vesicle or non-vesicular transport. Due to the strong hydrophobicity of squalene a non-vesicular transport appears to be more likely. Such mechanisms may include membrane contact or the involvement of helper proteins such as oxysterol binding proteins [3;4], their homologues, the so-called Osh proteins [5], or even sterol carrier proteins such as SCP1 and SCP2 [6]. However, squalene stored in LP also has to migrate from this subcellular site to its site of further conversion. In the yeast, the situation is puzzling insofar as the squalene converting enzyme, the squalene epoxidase Erg1p is present in LP but most likely in an inactive form [7]. Consequently, there may be the need for squalene delivery to the ER where enzymatically active Erg1p is located. Experiments using plants [8] addressed squalene mobilization from LP in some detail. Such experiment should also be possible with the yeast.

Simon *et al.* [9] and Lohner *et al.* [10] provided evidence that squalene can localize inside a membrane. Simon and coworkers suggested not axial position of squalene in the membrane bilayer. Lohner *et al.* [10] found out that squalene at a concentration of 6 mol % in phospholipid vesicles changed the lamellar-to-inverse-hexagonal phase transition with increasing the size of inverse hexagonal phase tubes. These authors argued that squalene must be stored in a most disordered region of the bilayer, most likely existing rather in a coiled than extended conformation and localizing to the interior of the bilayer. Our experiments using biological and model membranes extend this model insofar as squalene in the ER may rather adapt to a conformation close to ergosterol, whereas in the plasma membrane squalene might be coiled. This would explain increased permeability of plasma membrane and thus sensitivity against higher or lower pH, higher salt concentration or detergent addition.

Hauss *et al.* [11] reported that squalane, the hydrogenated form of squalene, was stored in the midplane of a membrane bilayer with a barrier function for proton flux. If that was true for squalene, low pH sensitivity would not occur in yeast cells accumulating squalene in the plasma membrane. It has to be taken into account, however, that Hauss *et al.* [11] worked with artificial membranes whereas our results presented here were obtained with biological membranes. In such biological membranes other components might have additional of compensating effects making interpretations more difficult. Thus, the effect of squalene in biological membranes seems is probably less dramatic. In any case, the membrane modulating role of squalene as described in this Thesis may be regarded as a novel biophysical property of this lipid.

Another function of squalene for biotechnological and pharmaceutical purposes discussed also in this Thesis is squalenoylation. This technique is used for facilitated drug delivery into cells such as in the case of gemcitabine-squalene. Squalenoylation helps to solubilize the drug, which can then easily penetrate through the plasma membrane and thus enter the cell. Squalene is then further metabolized to sterol [12]. It has not been shown, however, whether or not squalenoylation also occurs in nature. Formation of hybrids may improve the transport of hydrophilic components across membranes or to hydrophobic compartments such as LP. Even association of squalene with proteins may be envisaged. This would a special case for facilitated transport of proteins. Such scenarios, however, are pure speculation at this point.

In summary, this Thesis was a step forward in understanding the role of squalene as a lipid component and as a modulator of hydrophobic compartments in the cell. Future experiments addressing some of the aspects discussed above may lead to a more elaborate picture of the role of this lipid.

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# Curriculum Vitae


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| 2002 - 2007 | **Member of Student Parliament** |
| 2005 - 2007 | **Vice-President of Student Parliament**<br>*Communication with Students and Directory, organization of student balls, team-working* |
| 2005 - 2007 | **Senator in Academic Senate at Slovak University of Technology**<br>*Communication with directory of University, representation of students of Faculty in Academic Senate* |
| 2004 - 2007 | **Organizer at Beania Chemikov, University Student Ball**<br>*Organization of student balls, communication with sponsors* |
| 2002 –2005 | **Senator in Academic Senate at Faculty of Chemical and Food Technology Slovak University of Technology**<br>*Communication with directory of Faculty, representation of students in Academic Senate* |

**Publications:**

1. Spanova, M. , Czabany, T., Zellnig, G., Leitner, E., Hapala, I., Daum, G. (2010): Effect of Lipid Particle Biogenesis on the Subcellular Distribution of Squalene in the Yeast *Saccharomyces cerevisiae* Journal of Biological Chemistry 285, *6127-6133*

2. Spanova, M., Zweytick,D., Lohner, K., Leitner, E., Hermetter, A. and Daum, G. Influence of Squalene on Lipid Particle/Droplet and Membrane Organization in the Yeast *Saccharomyces cerevisiae*
*Manuscript in Preparation*

3. Spanova, M. and Daum, G.
Biochemistry and Biotechnology of Squalene
*Manuscript in Preparation*

**Oral presentations:**

1. M.Spanova, T. Czabany, G. Zellnig, D. Zweytick, K. Lohner, E. Leitner, I. Hapala and G. Daum
   Squalene in Yeast and its Effect on Lipid Particle Biogenesis
   FEBS Workshop Microbial Lipids: From Genomics to Lipidomics, Vienna, Austria, 13-15 May 2010

2. M. Spanova, T. Czabany, G. Zellnig, E. Leitner, I. Hapala and G. Daum
   Effect of Lipid Particle Biogenesis on the Subcellular Distribution of Squalene in the Yeast *Saccharomyces cerevisiae*
   3rd International Graz Symposium on Lipid and Membrane Biology, Graz, Austria, 18-20 March 2010

3. M. Spanova, T. Czabany, G. Zellnig, E. Leitner, I. Hapala and G. Daum
   Effect of Lipid Particle Biogenesis on the Subcellular Distribution of Squalene in the Yeast *Saccharomyces cerevisiae*
   3rd DocDay – NAWI Graz Doctoral School, Graz, Austria, 12th February 2010

4. M. Spanova, T. Czabany, G. Zellnig, E. Leitner, I. Hapala and G. Daum
   Lipid Particles as a Storage Compartment for Squalene in the Yeast *Saccharomyces cerevisiae*
   37th Annual Conference on Yeasts, Smolenice, Slovakia, 13-15 May 2009

**Co-Author in Lectures:**

1. G. Daum, K. Athenstaedt, A. Wagner, T. Czabany, K. Grillitsch, S. Rajakumari, M. Spanova, D. Zweytick and E. Ingolic (Invited Lecture)
   Formation and mobilization of neutral lipid depots in the yeast
   3rd European Federation of Biotechnology Conference on Physiology of Yeast and Filamentous Fungi, Helsinki, Finland, 13-16 June 2007

2. T. Czabany, A. Wagner, D. Zweytick, E. Ingolic, M. Spanova, I. Hapala and G. Daum
   Lipid particle variants from the yeast *Saccharomyces cerevisiae*
   35th Annual Conference on Yeast, Smolenice, Slovakia, 16-18 May 2007

3. G. Daum, T. Czabany, A. Wagner, M. Spanova, S. Rajakumari, K. Grillitsch and K. Athenstaedt
   Playing the yeast lipid game (Invited Lecture)
   8th Yeast Lipid Conference, Torino, Italy, 10-12 May 2007

**Poster presentations:**

1. M. Spanova, T. Czabany, G. Zellnig, E. Leitner, I. Hapala and G. Daum
   Lipid Particles as a Storage Compartment for Squalene in the Yeast *Saccharomyces cerevisiae*
   9th Yeast Lipid Conference, Berlin, Germany, 21-23 May 2009

2. M. Spanova, T. Czabany, E. Leitner, I. Hapala and G. Daum
   Squalene Storage and Subcellular Distribution in the Yeast *Saccharomyces cerevisiae*
   Joint Annual Meeting of ÖGBM, ÖGGGT, ÖGBT and ANGT, Graz, Austria, 21-24 September 2008

3. Z. Mrozova, T. Czabany, M. Spanova, M. Valachovic and I. Hapala
   Regulácia metabolizmu neutrálnych lipidov u kvasiniek *S. cerevisiae* (Regulation of neutral lipid metabolism in the yeast *S. cerevisiae*)
   21th Biochemical meeting of CSBMB a SSBMB , Ceske Budejovice, Czech Republic, 14-17 September 2008

4. M. Spanova, T. Czabany, E. Leitner, I. Hapala and G. Daum
   Squalene storage and subcellular distribution in the yeast *Saccharomyces cerevisiae*
   49th International Conference on the Bioscience of Lipids, Maastricht, The Netherlands, 26-30 August 2008

5. Z. Mrozova, T. Czabany, M. Spanova, M. Valachovic and I. Hapala
   The role of intracellular fatty acid synthesis in the control of triglyceride levels in the yeast *Saccharomyces cerevisiae*
   12th International Congress on Yeasts, Kyiv, Ukraine, 11-15 August 2008

6. T. Czabany, A. Wagner, D. Zweytick, E. Ingolic, M. Spanova, I. Hapala and G. Daum
   Lipid particle variants from the yeast *Saccharomyces cerevisiae*
   32nd FEBS Congress "Molecular Machines", Vienna, Austria, 7-12 July 2007

7. T. Czabany, A. Wagner, D. Zweytick, E. Ingolic, M. Spanova, I. Hapala and G. Daum
   Lipid particle variants from the yeast *Sacchaomyces cerevisiae*
   8th Yeast Lipid Conference, Torino, Italy, 10-12 May 2007

8. Z. Mrozova, T. Czabany, M. Spanova, G. Daum and I. Hapala
   Oxygen effects in storage lipid biogenesis in the yeast *Saccharomyces cerevisiae*
   8th Yeast Lipid Conference, Torino, Italy, 10-12 May 2007

9. Z. Mrozova, T. Czabany, M. Spanova, M. Valachovic, M. Certik and I. Hapala
   "Slim yeast on high-fat diet": *Saccharomyces cerevisae* as a model in obesity research?
   34th Annual Conference on Yeasts, Smolenice, May 2006