Genetic engineering and molecular characterization of yeast strain expressing hybrid human-yeast squalene synthase as a tool for anti-cholesterol drug assessment

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Running headline: Analysis of human-yeast SQS

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Abstract

Aims: The main objective of the study is molecular and biological characterization of the human-yeast hybrid squalene synthase, as a promising target for treatment of hypercholesterolemia.

Methods and Results: The human-yeast hybrid squalene synthase, with 67% amino acids, including the catalytic site derived from human enzyme, was expressed in S. cerevisiae strain deleted of its own squalene synthase gene. The constructed strain has a decreased level of sterols compared to the control strain. The mevalonate pathway and sterol biosynthesis genes are induced and the level of triacylglycerols is increased. Treatment of the strain with rosuvastatin or zaragozic acid, two mevalonate pathway inhibitors, decreased the amounts of squalene, lanosterol and ergosterol, and up-regulated expression of several genes encoding enzymes responsible for biosynthesis of ergosterol precursors. Conversely, expression of the majority genes implicated in the biosynthesis of other mevalonate pathway end-products, ubiquinone and dolichol, was down-regulated.

Conclusions: The S. cerevisiae strain constructed in this study enables to investigate the physiological and molecular effects of inhibitors on cell functioning.

Significance and Impact of the Study: The yeast strain expressing hybrid squalene synthase with the catalytic core of human enzyme is a convenient tool for efficient screening for novel inhibitors of cholesterol-lowering properties.

Keywords: mevalonate pathway, squalene synthase, sterol biosynthesis inhibitors, yeast expression system, heterologous proteins
Introduction

Conversion of HMG-CoA to mevalonate is an early step in the cholesterol biosynthesis. Mevalonate is also a precursor for biologically important nonsteroidal isoprenoids, for example: dolichol, ubiquinone, isopentenyl tRNA and prenylated proteins, which have an important role in the regulation of cellular processes. Statins, by competitive inhibition of the HMG-Co reductase (HMGR) reduce endogenous cholesterol production, increase the number of LDL receptors and thereby lower the serum cholesterol level (Opie 2015). Although statin therapy is commonly assumed to be well tolerated, serious adverse effects have been reported. Therefore, it is postulated that some statins side effects may be due to the fact that statins suppress all post mevalonate biosynthesis steps including non-steroidal isoprenoids (Charlton-Menys and Durrington 2008). Probably inhibition of HMGR, a key regulator of the mevalonic acid pathway, causes not only decrease of cholesterol biosynthesis, but also disturbances in the synthesis of other molecules like isopentyl diphosphate, farnesyl diphosphate and geranylgeranyl diphosphate. Hence, there is a need for new medicines to lower cholesterol levels, without any serious adverse reactions and also effective against hypercholesterolemia (Seiki and Frishman 2009).

In both humans and yeast the mevalonate pathways are highly conserved (Fig. 1). They are identical till the zymosterol formation with the end product in human cells being cholesterol, and ergosterol in yeast. The use of yeast as a host for the expression of heterologous proteins has become increasingly popular in recent years (Nielsen 2009). As an eukaryote, S. cerevisiae has many of the advantages of higher eukaryotic expression systems such as protein processing, protein folding and posttranslational modifications, while being as easy to manipulate as the bacteria E. coli. As a model of
fundamental cellular processes and metabolic pathways of the human, yeast have improved the understanding and facilitated the molecular analysis of many disease genes (Menacho-Márquez and Murguía 2007; Ruggles et al. 2014). Comparative genomics studies have shown that 40% of yeast proteins show homology to at least one human protein and 30% of genes involved in human disease pathology have an ortholog in yeast (Sturgeon 2006). Also, many regulatory pathways are conserved between yeast and humans. Therefore, yeast emerge as an attractive model in drug development studies like identification of new drug targets, target-based and non-target based drug screening and analysis of the cellular effects of drugs (Hughes 2002; Wysocka-Kapcinska et al. 2009; Marjanovic et al. 2010).

In our study, squalene synthase (SQS), the first dedicated enzyme of sterol biosynthesis, has been chosen as a promising target for treatment of hypercholesterolemia. Squalene synthase catalyzes the conversion of trans-farnesyl diphosphate to squalene, the first specific step in the cholesterol biosynthetic pathway, and is responsible for the flow of metabolites into either the sterol or the non sterol branch of the pathway (Do et al. 2009). Squalene synthase inhibitors (SSIs) reduced hepatic cholesterol biosynthesis by the induction of hepatic LDL receptors in a similar way to statins (Charlton-Menys and Durrington 2007). Several classes of squalene synthase inhibitors have been studied as potent inhibitors of squalene synthase (Kourounakis 2011). For example, the fungal metabolite zaragozic acids or sualestatins have been investigated as potent inhibitors of squalene synthase in human and other species (Liu et al. 2012). SSIs do not cause myotoxicity, and when administered together with a statin, reduce the statin-induced myotoxicity (Nishimoto et al. 2007). On the other hand, most of SSIs failed to pass clinical phase I/II trials because of their
hepatoxicity (Liao 2011). Nevertheless, squalene synthase is still considered as a promising target for therapeutic molecules that could decrease cholesterol level without affecting other isoprenoids.

Yeast and human squalene synthase amino acid sequences show 36% amino acid identity and 57% similarity. Apart from the amino and carboxyl termini, which are not similar, the conservation of sequences responsible for the catalytic activity of the protein is very high (Robinson et al. 1993). The activity of squalene synthase is essential for cell growth in yeast (Jennings et al. 1991), and a deletion of the ERG9 gene encoding yeast SQS is lethal. It has been shown previously that human FDFT1 gene encoding hSQS expressed in yeast cells lacking native squalene synthase (erg9Δ) fails to restore viability of the defective strain (Robinson et al. 1993; Soltis et al. 1995).

In this study, we present molecular and biological characterization of the human-yeast hybrid squalene synthase, containing 67% of human SQS including the catalytic site. Moreover, we analyse the impact of selected mevalonate pathway inhibitors on the cell metabolism. Proposed by us, yeast model expressing hybrid squalene synthase with the catalytic core of human SQS allows rapid, inexpensive, highly efficient screening of in silico designed substances of cholesterol-lowering properties. Its application for such screening will result in elimination of molecules of less effectiveness or causing adverse effect.

**Materials and methods**

**Media and growth conditions**
Saccharomyces cerevisiae strains were cultivated in standard YP medium (1% Bacto-
yeast extract, 1% Bacto-peptone) containing 2% dextrose (YPD), 3% glycerol (YPGly),
or 3% ethanol (YPEtOH). Synthetic SD minimal medium (0.67% Bacto-yeast nitrogen
base, 2% dextrose) supplemented with a required synthetic complete amino acids drop-
out mixture (Sunrise Science Products, San Diego, CA, USA) was used for selection of
yeast cells bearing plasmids. Solid media were prepared by the addition of 2% Bacto-
agar. URA3 counter-selection of yeast was carried out on SD plates supplemented with
0.1% 5-fluoroorotic acid (5-FOA; Sigma-Aldrich, St. Louis, MO, USA) and required
amino acids. To select cells containing the kanMX4 cassette, YPD was supplemented
with geneticin (G418 sulphate, Sigma-Aldrich) at the concentration of 200 μg ml⁻¹. To
induce membrane permeability, nystatin (Sigma-Aldrich) was added to YPD to the final
concentration of 5 μg ml⁻¹. Rosuvastatin stock solution (10 mg ml⁻¹) was prepared by
extracting an active substance from Crestor (AstraZeneca AB, Sweden), as described
previously (Maciejak et al. 2013). Zaragozic acid (Sigma-Aldrich) was dissolved in
DMSO to obtain 10 mg ml⁻¹ stock solution. Yeast cells were grown aerobically either in
liquid or on solid medium, at 28°C. To perform growth curves overnight cultures were
diluted to the concentration of 0.5x10⁷ cells/ml and cells were grown in SD-HIS
medium at 28°C for 14 hours. Optical density (OD₆₀₀) measurements were done after
every two hours of cultivation. Each culture was assayed in triplicate and the results
were averaged.

Homology modeling of human and yeast SQS structure

Models of yeast and hybrid SQS fragments (34-383 and 35-376, respectively) were
obtained using SYBYLx2.1, TRIPOS Inc., on the basis of human SQS structure (PDB...
entry 3VJ8) (Liu et al. 2012). The model structures were subjected to staged energy minimization using AMBERFF99 forcefield, 100 steps. The model structure of zaragozic acid complexed with hybrid SQS was obtained on the basis of the structure of human SQS in complex with zaragozic acid (3VJC), (Liu et al. 2012).

**DNA manipulations and plasmid construction**

Standard protocols were used for all DNA manipulations (Green and Sambrook 2012). The *Escherichia coli* strain XL1-Blue MRF’ (Stratagene, La Jolla, CA, USA) was used for cloning and propagation of plasmids. The sequences of all primers and methodology of plasmid construction are shown in the Supporting Information, Table S1 and Figure S1, respectively. The *ERG9* gene, encoding yeast SQS, was amplified with BamERG9 and ERG9SalI primers and ligated into pGEM-T Easy vector (Promega, Madison, WI, USA). The FDFT1 cDNA, encoding human SQS, was amplified with 1FDFTbam and 2FDFTeco primers and ligated into pGEM-T Easy. The resulting plasmids served as templates to construct hybrid squalene synthase (*HYB*). Both plasmids were digested with MscI and SalI and the *ERG9* MscI-SalI fragment was subcloned into MscI-SalI cut FDFT1/pGEM. Next, the resulting plasmid was digested with MscI and ligated with the MscI-MscI fragment of FDFT1 gene to form *HYB*/pGEM. Tagging of *HYB* sequence with 6HA was carried out by PCR-amplification of *HYB* from *HYB*/pGEM using HindHYB and HYBSalI primers and cloning into HindIII-SalI digested pYM16 (Janke et al. 2004). Next, the promoter of *ERG9* gene, amplified with HinpERG9 and pERG9Hin primers, was introduced into HindIII site of *HYBHA*/pYM16 plasmid. Finally, *P_{ERG9-HYBHA}* was cloned into NotI and SmaI cut pRS313 (Sikorski and Hieter 1989). The analogous vector carrying *ERG9* tagged with 6HA was constructed by PCR-
amplification of the $P_{ERG9}$-$ERG9HA$ sequence with NotI-ERG9 and ERG9Xmal primers from genomic DNA of the $ERG9HA$ strain (construction details in the Supplementary Material). The amplified $P_{ERG9}$-$ERG9HA$ fragment was cloned into NotI-Xmal digested pRS313.

Construction of yeast strains expressing recombinant squalene synthase

The *Saccharomyces cerevisiae* BY4741 and heterozygous diploid $erg9\Delta/ERG9$ in the background of BY4743 (EUROSCARF, Frankfurt, Germany) were used as the parental strains for yeast strains constructed in this study. All yeast strains are listed in the Supporting Information, Table S2. Yeast transformation was performed according to Gietz and Woods (2002). Heterozygous diploid $erg9\Delta/ERG9$ was transformed with $P_{GAL1}$-$ERG9$-pYES2 plasmid, sporulated and tetrads were dissected and cultured on YPD plates. Haploid $erg9\Delta\ [P_{GAL1}$-$ERG9]$ was selected after replica plating on YPD supplemented with geneticin and SD-URA. This haploid served for further constructions of strains expressing yeast and hybrid squalene synthase. In order to construct the $erg9\Delta\ [P_{ERG9}$-$HYBHA]$ strain expressing human-yeast hybrid squalene synthase, the $P_{ERG9}$-$HYBHA$/pRS313 plasmid was transformed into $erg9\Delta\ [P_{GAL1}$-$ERG9]$ strain. Subsequently, the $P_{GAL1}$-$ERG9$/pYES2 plasmid was lost on SD-HIS plates containing 5-FOA to obtain the investigated strain. The $erg9\Delta\ [P_{ERG9}$-$ERG9HA]$ control strain was obtained by transformation of $erg9\Delta\ [P_{GAL1}$-$ERG9]$ strain with $P_{ERG9}$-$ERG9HA$/pRS313. Next, the $P_{GAL1}$-$ERG9$/pYES2 plasmid was lost on SD-HIS plates containing 5-FOA.

Lipid extraction
Yeast strains were grown in SD-HIS medium with or without an inhibitor, starting from 0.5x10^7 cells/ml. After 10 hours, cells were spun down, pellets were weighed and lipid extraction was performed according to the procedure described by Folch et al. (1957) with minor changes, namely 16 μg cholestanol/sample was added as an internal standard. Cells were homogenized with chloroform/methanol (1:1) and 0.063-0.200 mm Silica Gel 60 (Merck, Darmstadt, Germany) by 5 min vortexing and overnight shaking. Homogenates were spun down, solvent was removed to new tubes and remaining pellets were re-extracted twice for 5 hours. Extracts were pooled and washed three times with 1/5 volume of 0.9% NaCl solution. The lower, chloroform phase containing lipids was transferred to new tube and the solvent was evaporated under a stream of nitrogen and dissolved in chloroform:methanol to yield crude lipid extract. For sterol analysis an aliquot of crude lipid extract was dried and hydrolyzed after supplementation with ethanol/toluene/water (82:100:15) solution with 7.5% KOH (w/v) for 2 hours at 100°C. Lipids were extracted with equal volume of diethyl ether, the upper phase was transferred to a new tube and the lower phase was re-extracted twice with diethyl ether, then the extracts were pooled and evaporated and lipids were dissolved in 500 μl hexane.

**GC/MS analysis of lipids**

The Agilent 5975C GC/MSD (a gas chromatograph and a mass spectrometer detector of Agilent Technologies, Santa Clara, CA) equipped with a 30 m long HP-5ms column, with 0.25 mm inner diameter, and 0.25 μm stationary phase film thickness were used. 1 μl of lipid sample (hexane extract) was injected and the column temperature was set at 150°C for 5 min, next it was increased to 300°C with the ramp of 5°C/min and the final temperature was set at 300°C for 30 min. Helium was used as a carrier gas and the flow
rate was set on 1 ml/min. The scan mode of 33-600 m/z was used to monitor mass
spectra. Sterols were identified by comparing their spectra with the those of the NIST
under the signals of sterol and of internal control (cholestanol) served to calculate the
amount of sterol.

**TLC lipid analysis**

A thin layer chromatography (TLC) was carried out for crude lipid extract. Samples
were evaporated and dissolved in the appropriate amount of chloroform so that an equal
concentration of total lipids in each sample was achieved. 50 µl of each sample
corresponding to 60 µg of wet yeast mass spotted on a TLC plate (Silica Gel 60 F254
0.2 mm) and the plate was developed with petrol ether/diethyl ether/acetic acid
(90:10:1) as a mobile phase. Subsequently, the plate was dried and lipids were
visualized by iodine vapor. Densitometric analysis of freshly stained TLC
chromatogram, covered with a glass plate to avoid iodine desorption, was performed to
estimate the relative lipid content (ImageJ2x software, Java-based image processing
program, developed at the National Institutes of Health). Lipids were identified by
comparison with external standards.

**Quantitative real-time RT-PCR**

Yeast strains were grown in SD-HIS medium with or without an inhibitor, starting from
0.5x10^7 cells/ml. Each culture was prepared in three independent replicates. After 10
hours, cells were harvested, homogenized with MagNA Lyser Instrument (Roche
Diagnostics GmbH, Germany) and total RNA was isolated with MagNA Pure Compact
Instrument (Roche) according to the manufacturer’s instruction. cDNA was synthesized with QuantiTect Reverse Transcription Kit (Qiagen, Hilden, Germany), following the instruction. Primers used for RT-qPCR are presented in the Supporting Information, Table S3. RT-qPCR was carried out in 96-well plates using the LightCycler FastStart DNA Master SYBR Green I and the LightCycler 480 System (Roche). Each sample was run in triplicate. In order to calculate the relative expression ratio of genes between experimental and control samples, the Pfaffl model (Pfaffl 2001) was used and calculations were carried out in the REST-MCS v2 software tool. The expression data were normalized to the reference gene ACT1. All experiments were performed according to the MIQE guidelines (Bustin et al. 2009).

Results

Human and yeast SQS models show different structures

A comparison of the modeled yeast SQS structure with that of the experimentally derived human SQS structure of the human and yeast SQS proteins showed no major differences around the active center, but significant structural alterations between the yeast fragments N113-D119 and D161-T171, and the corresponding human SQS regions M112-D118 and D159-S164 (Supporting Information, Fig. S2). These fragments are located in close proximity to the central cavity, so one may expect differences in the binding of inhibitors to the human and yeast squalene synthases. Taking into consideration all the above-mentioned aspects, a hybrid squalene synthase with the catalytic core of human SQS was constructed in this study. A model structure of the hybrid SQS was compared with the structure of human SQS. On the basis of
published data (Liu et al. 2012; Liu et al. 2014; Shang et al. 2014) it was established that residues constituting the active center of human SQS are located in fragment 35 - 327 of the enzyme. Superposition of backbone atoms of the 35 -327 fragment gave an RMSD (root mean square deviation) of about 0.1 Å between the human and the hybrid enzyme, what means that both structures are virtually identical in that region. Differences between both structures appear at the C-terminal part of the protein, which is far away from the active site. Fig. 2 presents a comparison of the main chain course of the human and the hybrid SQS. The part of human SQS structure (P354 - K358) substantially different from the hybrid SQS is also shown.

HYBHA hybrid gene restores erg9Δ viability

*S. cerevisiae* erg9Δ strain bearing the FDFT1-ERG9 hybrid gene (HYBHA) was constructed similar to Robinson et al. (1993). The hybrid squalene synthase comprised 67% of human SQS aa sequence (1-296 amino acids) including the catalytic site, and 33% of yeast SQS sequence (304-444 amino acids), which included transmembrane domain necessary for the attachment of protein to the membranes of endoplasmic reticulum (Supporting Information, Fig. S3). Importantly, in our study the squalene synthase hybrid gene was expressed from the native *ERG9* promoter, and was fused on its 3’ end with hemagglutinin (HA) tag sequence. The P*ERG9*-HYBHA construct was cloned into centromeric pRS313 vector and was expressed in yeast erg9Δ strain. Control strains erg9Δ bearing P*ERG9*-ERG9HA on pRS313 and BY4741 transformed with an empty pRS313 vector were also constructed.

On the contrary to the full-length human FDFT1 gene, the human-yeast hybrid gene HYBHA was able to restore viability of erg9Δ yeast cells. All tested strains were
growing equally on a complete medium, a minimal (histidine lacking) medium and glycerol or ethanol containing medium at 28°C (Fig. 3a). No statistically significant difference between the growth curves was observed (Fig. 3b).

Additionally, all strains were tested on nystatin supplemented medium (Fig. 3c). Nystatin is a polyene macrolide antibiotic which forms ion channels on plasma membrane, resulting in cation leakage and cell death. The nystatin activity was shown to be significantly affected by the ergosterol membrane’s molar fraction (Kristanc et al. 2014). The HYBHA expressing strain was resistant to nystatin, which might be due to lowered content of ergosterol in plasma membranes.

Sterol biosynthesis is disturbed in HYBHA expressing strain

To evaluate the level of squalene and sterol in yeast cells, gas chromatography/mass spectrometry (GC/MS) analysis of lipids was performed. The content of squalene, lanosterol and ergosterol was diminished in HYBHA expressing strain, and it reached the level of 10.9% (p=0.035), 43.7% (p=0.094) and 82.3% (p=0.342) respectively, in comparison to the control strain (Fig. 4a).

Since the sterol biosynthesis was impaired in the erg9Δ [P_{ERG9-HYBHA}] strain, sterol storage forms might be modified as a result of diminished sterol content. Sterols are stored in yeast cells in the form of esters and they are located in lipid particles (LP) together with triacylglycerols (Czabany et al. 2007). As tested by semiquantitative thin layer chromatography method (TLC), the erg9Δ [P_{ERG9-HYBHA}] strain showed a different profile of lipids than the control (Fig. 4b). Both, the level of free ergosterol and also ergosterylesters was reduced. On the contrary, the level of triacylglycerols and free fatty acids was increased.
mRNA expression level of isoprenoid biosynthesis pathway genes is changed in

*HYBHA* expressing strain

Quantification of mRNA for genes encoding selected enzymes (shown in Fig. 1) from
the sterol and non-sterol isoprenoid biosynthesis pathways was performed by RT-qPCR.

Gene expression levels in the *erg9A [P_EROG-HYBHA]* strain cultured at 28°C were
related to the expression of respective genes in *erg9A [P_EROG-ERG9HA]* strain grown at
the same temperature (Fig. 5a and b). The expression of all the tested genes of sterol
biosynthesis was induced (Fig. 5a), except for the *HMG2* gene which is one of the yeast
paralogues coding for HMG-CoA reductase. The expression of genes coding for
enzymes of other mevalonate-end products was only slightly changed in the *erg9A
[P_EROG-HYBHA]* strain (Fig. 5b), the majority of them were down-regulated.

The levels of squalene and sterols are lowered in *HYBHA* expressing strain
cultured with inhibitors

The constructed *erg9A [P_EROG-HYBHA]* strain was used in further tests of two inhibitors
of the mevalonate pathway: zaragozic acid – inhibitor of squalene synthase, and
rosuvastatin – inhibitor of HMG-CoA reductase. They were chosen to compare the
effect of the inhibition of the mevalonate pathway at the level of SQS and HMGR. To
this end, median lethal dose of inhibitors (LD₅₀) was assessed at permissive temperature
of 28°C. The final concentration of 1.3 μmol l⁻¹ for zaragozic acid and 22.8 μmol l⁻¹ for
rosuvastatin were needed for 50% growth inhibition after 10 hours of cultivation.

To determine the influence of zaragozic acid and rosuvastatin on squalene and
sterol content in *HYBHA* expressing strain, GC-MS analysis was performed for lipids
isolated from cells grown in the presence or absence of the respective inhibitor. As
expected, both inhibitors decreased the levels of squalene, lanosterol and ergosterol. The effect of rosuvastatin on sterol biosynthesis inhibition was higher than that of zaragozic acid at the same toxic dose of LD$_{50}$. The amount of lipids was reduced to: 4.6% vs 17.5% (p=0.032) for squalene, 4.3% vs 21.6% (p=0.005) for lanosterol and 60% vs 80.8% (p=0.093) for ergosterol, by rosuvastatin and zaragozic acid, respectively (Fig. 6).

**Inhibition of the mevalonate pathway alters the expression of selected genes**

Rosuvastatin and zaragozic acid act at different points of the mevalonate pathway, might differently affect the availability of FPP. Rosuvastatin, by inhibition of the early step of the pathway, diminishes the cellular level of farnesyl diphosphate (Liao 2002). On the contrary, blocking the major FPP utilizing branch at the level of squalene synthase results in elevated FPP availability. Taking this into account, the expression of mevalonate pathway genes, especially genes from side branches in response to inhibitor, was followed. RT-qPCR analysis was performed for the erg$9\Delta$ [P$_{ERG9}$-HYBHA] strain cultured with or without the respective inhibitor.

As was expected, genes of the early mevalonate pathway and sterol biosynthesis branch were up-regulated in response to both inhibitors (Fig. 7a, left diagram). On the contrary, the expression of genes of other isoprenoid pathway-end products, below the FPP-branching was mostly decreased, except for MOD5 (Fig. 7a, right diagram).

Surprisingly, the tendency of changes in mRNA levels was consistent for both inhibitors. In order to check whether increased doses of inhibitors would enhance the changes in gene expression, RT-qPCR was performed for the HYBHA expressing strain inhibited with LD$_{70}$ dose of rosuvastatin or zaragozic acid. Indeed, although the general
trend seemed similar, the rate of up- or down-regulation was better pronounced (Fig. 7b) than in case of LD₅₀.

The mRNA level of hybrid squalene synthase was up-regulated in response to treatment with inhibitors (Fig. 7a and b). Also the level of SQS protein was slightly increased by both rosuvastatin and zaragozic acid (Supporting Information, Fig. S4).

Discussion

Since a comparison of modeled structures of yeast and human squalene synthases revealed substantial conformational differences close to the central cavity, a hybrid SQS was constructed. The human-yeast hybrid squalene synthase, containing 67% of human SQS aa, including the catalytic site, was expressed in Saccharomyces cerevisiae erg⁹Δ strain in order to define the utility of yeast as a tool for screening human SQS inhibitors.

The ERG⁹ gene expression in yeast has been reported to undergo complex regulation, both positive and negative, by diverse factors through cis-elements in the promoter (Kennedy et al. 1999; Kennedy and Bard 2001). Using the native ERG⁹ promoter to drive expression of the hybrid SQS gene we ensured its proper regulation and expression. This is crucial because squalene synthase constitutes an important point of the mevalonate pathway, second to HMG-CoA reductase, that is regulated by a sterol feedback mechanism and is responsible for directing substrates to different branches of the pathway.

Although during standard growth conditions the erg⁹Δ [P₄ERG⁹–HYBHA] strain behaves like a control strain, it is resistant to nystatin. Nystatin was previously reported to be specific for ergosterol in yeast cell membranes (Walker-Caprioglio 1989), and sterol mutants (e.g. ERG⁷, ERG⁶, ERG⁵) are resistant to nystatin (SGD Database,
http://www.yeastgenome.org). This might suggest that qualitative or quantitative changes in sterol content of yeast cellular membranes are correlated with resistance to nystatin. GC-MS analysis, indeed, showed that the strain expressing hybrid SQS has diminished amount of squalene, lanosterol and ergosterol.

Mevalonate pathway regulation is very complex and only partially understood, however, it is known to proceed at multiple levels, such as transcriptional activation and repression, maintaining protein stability or stimulating protein degradation, and it involves sterol and non-sterol intermediates of the pathway. In general, genes of the early part of mevalonate pathway as well as genes of sterol biosynthesis branch are overexpressed in the HYBHA expressing strain, except the MG2 gene, which is slightly down-regulated. We suspect that the overexpression is caused by a sterol-mediated feedback response, which has previously been reported both in mammalian and yeast cells (Dimster-Denk et al. 1994; Dimster-Denk et al. 1999).

The second important regulation point of the mevalonate pathway resides at the level of FPP, which is considered as the common precursor for numerous isoprenoids. The expression of genes placed in the intersectional pathways (shown in Fig. 1) is generally decreased in the erg9Δ [P_ERG9–HYBHA] strain. Perhaps the underlying reason is an effort of cells to redirect the flux of FPP to the impaired sterol biosynthetic pathway or a rescue mechanism in response to possibly increased level of farnesyl diphosphate.

In summary, the yeast strain expressing the hybrid SQS contains a lower level of sterols than the control strain although the amount of sterols is sufficient to maintain normal growth at standard growth conditions. The diminished level of sterols triggers
an adaptive response of the cell, which includes an overexpression of the mevalonate pathway and sterol biosynthesis branch genes.

We tested two inhibitors acting at different enzymes of the mevalonate pathway, namely rosuvastatin and zaragozic acid that inhibit HMG-CoA reductase and squalene synthase, respectively. A rationale for this selection was a possible contrapositive effect on the pool of farnesyl diphosphate exerted by HMG-CoA and SQS inhibitors.

Inhibition of mevalonate pathway either at the level of HMGR or SQS affects the synthesis of squalene and finally sterol production in the erg9Δ[P\text{\textit{ERG9}}-HYBHA] strain. The most significant reduction was seen for ergosterol biosynthetic precursors, such as squalene and lanosterol. On the contrary, the last product of the branch, ergosterol, was only slightly decreased, which might be caused by efficient conversion of precursors and mobilisation of sterol stored in lipid particles. Rosuvastatin appears to impair sterol biosynthesis to a greater extent than zaragozic acid when compared at LD_{50}. Sensitivity of the erg9Δ[P\text{\textit{ERG9}}-HYBHA] strain to mevalonate pathway inhibitors proves that this strain may be successfully used for SQS inhibitor screening.

Rosuvastatin and zaragozic acid induced the expression of genes of the early steps of mevalonate pathway (except the HMG2 gene) and genes specific for sterol synthesis. The enhanced expression was most likely related to the feedback response triggered by decreased amounts of sterol. A similar effect on the expression of genes along the mevalonate pathway in yeast observed during HMG-CoA and SQS inhibition was reported by Dimster-Denk et al. (1999) and Kuranda et al. (2010). Due to an opposite effect of the inhibition of HMGR and SQS, expected on FFP level, we followed the mRNA levels of genes from the branching pathways for rosuvastatin and zaragozic acid blocks. Inhibition of HMGR leads to a decreased amount of mevalonic
acid and depletion of downstream products along with farnesyl diphosphate (Liao 2002), which becomes less available for enzymes utilizing FPP. On the contrary, the SQS block results in an elevated FPP level (Bergstrom et al. 1993), that may be more accessible for FPP-consuming enzymes. We observed negligible effect of the inhibition on BTS1 and RER2 expression, but the levels of RAM1, COQ1 and COX10 mRNA were diminished in both tested strains. However, the directions of expression changes for specific genes were consistent for both inhibitors, which is most likely related to the influence of deficient sterols. Also the expression of other genes in branching pathways was repressed, such as ubiquinone biosynthesis genes (COQ3, CAT5), dolichyl phosphate biosynthesis gene (SEC59) and the gene coding for a protein that undergoes farnesylation, RAS1. The only genes that were up-regulated were COQ2 and MOD5, of which the first is involved in ubiquinone biosynthesis and the second is responsible for tRNA prenylation process. The alterations in the expression levels were even higher when the growth of HYBHA bearing strain was inhibited by 70% what indicates that the constructed strain demonstrates a dose – response effect.

Saccharomyces cerevisiae model that has been engineered in this study is a convenient tool for high-throughput screening for molecules designed to inhibit the catalytic site of human SQS. Moreover, the yeast model allows to study the physiological and molecular effects of tested molecules on the cell, such as mRNA and protein expression levels and lipid profile.

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Conflict of Interest: No conflict of interest declared.
References


Figure legends


**Figure 2** Structural comparison of human and hybrid SQS. Human protein is in green, hybrid in blue (light blue for human part, dark blue for yeast part). Active center is highlighted in yellow. Zaragozic acid is presented as sticks. Arrows indicate main differences between human and hybrid SQS structures.

**Figure 3** The HYBHA hybrid gene complements the *ERG9* gene. (a) Serial dilutions of the BY4741 strain bearing the empty vector, and both *ERG9HA* and *HYBHA* expressing
strains were spotted on YPD, minimal, glycerol and ethanol medium. Strains were grown at 28°C. (b) The growth curve of ERG9HA and HYBHA expressing strains grown in minimal liquid medium (without histidine) for 14 hours at 28°C. (■) erg9Δ [P_{ERG9}ERG9HA] and (○) erg9Δ [P_{ERG9-HYBHA}]. (c) The HYBHA expressing strain is resistant to nystatin. Serial dilutions of the tested strains were spotted on YPD and YPD supplemented with nystatin. Strains were grown at 28°C.

**Figure 4** Analysis of lipids. (a) The content of squalene and sterols in the HYBHA strain is diminished in comparison to the control strain. Strains were cultured in minimal (histidine lacking) medium at 28°C for 10 hours and lipid content was analyzed by GC/MS. (□) erg9Δ [P_{ERG9-ERG9HA}]; (■) erg9Δ [P_{ERG9-HYBHA}] and (*) p≤0.05. (b) The lipid profile is changed in the HYBHA strain comparing to the control strain. Strains were cultured at 28°C for 10 hours, lipids were extracted and separated on TLC plates. Numbers represent the ratio of the lipid in HYBHA expressing strain with respect to the control strain. One representative analysis of three independent TLC experiments is shown. (E) erg9Δ [P_{ERG9-ERG9HA}]; (H) erg9Δ [P_{ERG9-HYBHA}]; (SQ) squalene; (SE) sterol esters; (TAG) triacylglycerols; (FFA) free fatty acids and (ERG) ergosterol.

**Figure 5** mRNA levels of selected genes encoding enzymes of sterol and nonsterol biosynthesis pathways. (a) and (b) The expression of genes in the erg9Δ [P_{ERG9-HYBHA}] strain relative to the expression in the control strain cultured at 28°C. mRNA levels of genes coding for enzymes of the mevalonate pathway (1) above FPP and (2) sterol biosynthesis are indicated on the right panel. mRNA levels of genes coding for enzymes that participate in (3) protein prenylation, (4) ubiquinone biosynthesis, (5)
dolichol biosynthesis, (6) heme A biosynthesis, and (7) tRNA prenylation are indicated on the left panel. Expression data were normalized to ACT1 gene. Results are shown as a log2 of relative expression. (*) p≤0.05 and (**) p≤0.001.

Figure 6 The reduction of squalene and sterol content in HYBHA expressing strain is dependent on the type of inhibitor used. The erg9Δ [P_{ERG9-HYBHA}] strain was cultured in minimal (histidine lacking) medium at 28°C for 10 hours with (□) rosuvastatin or (■) zaragozic acid. % of sterol concentration in comparison to the “no treatment” group.

Figure 7 The expression of genes in the erg9Δ [P_{ERG9-HYBHA}] strain exposed to the inhibitor relative to the expression in unexposed cells. Yeast cells were cultured at 28°C for 10 hours in the presence or absence of the respective inhibitor at the dose of (a) LD₅₀ or (b) LD₇₀. mRNA levels of genes coding for enzymes of the mevalonate pathway (1) above FPP and (2) sterol biosynthesis branch are indicated on the right panel. mRNA levels of genes coding for enzymes that participate in (3) protein prenylation, (4) ubiquinone biosynthesis, (5) dolichol biosynthesis, (6) heme A biosynthesis, and (7) tRNA prenylation are indicated on the left panel. Expression data were normalized to ACT1 gene. Results are shown as a log2 of relative expression. (□) rosuvastatin; (■) zaragozic acid; (*) p≤0.05 and (**) p≤0.001.
Supporting Information

Table S1 Primers used for plasmid construction
Table S2 Yeast strains used in this study
Table S3 Sequences of primers used in real-time PCR

Supporting Materials and methods: Construction of the ERG9HA strain

Supporting Materials and methods: Immunodetection of HA-tagged squalene synthase

Figure S1 The methodology of plasmid construction. (a) Construction of hybrid squalene synthase coding sequence (HYB). (b) Construction of P_ERG9–HYBHA/pRS313 plasmid.

Figure S2 Human SQS ribbon model. DXXED conserved motifs are highlighted in light blue and loops S51-F54 and V314-K318 in dark blue (Liu et al. 2014). Changes in the main chain course for yeast SQS are shown in red; non conserved residues of N113-D119 and D161-T171 fragments are shown as sticks.

Figure S3 Alignment of yeast (Y) and human (H) SQS amino acid sequences. The hybrid human-yeast SQS protein consists of amino acid residues 1-296 of human SQS and residues 304-444 of yeast SQS. Arrow indicates the exchanged regions. Stars indicate identical amino acid residues. Sequences were aligned using the COBALT server (Papadopoulos and Agarwala 2007).

Figure S4 The steady-state squalene synthase protein (SQS-HA) level in the erg9A [P_ERG9–HYBHA] strain.


(a) [Image of agar plates with yeast colonies]

(b) [Graph showing yeast growth over time]

(c) [Image of agar plates with yeast colonies in different conditions]

ERG9 [ ]

\[ \begin{align*}
| \text{P}_{\text{ERG9-ERG9HA}} & \mid \\
| \text{P}_{\text{ERG9-HYBHA}} & \mid
\end{align*} \]

YPD - HIS - YPGly - YPETOH

\[ 10^9 \text{ cells/ml} \]

\[ \text{time [h]} \]

YPD | nystatin 5\mu g ml\(^{-1} \)