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RESEARCH ARTICLE

The *YTA7* gene is involved in the regulation of the isoprenoid pathway in the yeast *Saccharomyces cerevisiae*

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Keywords

YTA7; *ERG20*; isoprenoid pathway; lovastatin; zaragozic acid.

Abstract

The isoprenoid pathway in yeasts is important not only for sterol biosynthesis but also for the production of nonsterol molecules, deriving from farnesyl diphosphate (FPP), implicated in *N*-glycosylation and biosynthesis of heme and ubiquinones. FPP formed from mevalonate in a reaction catalyzed by FPP synthase (Erg20p). In order to investigate the regulation of Erg20p in *Saccharomyces cerevisiae*, we searched for its protein partners using a two-hybrid screen, and identified five interacting proteins, among them Yta7p. Subsequently, we showed that Yta7p was a membrane-associated protein localized both to the nucleus and to the endoplasmic reticulum. Deletion of *YTA7* affected the enzymatic activity of *cis*-prenyltransferase (the enzyme that utilizes FPP for dolichol biosynthesis) and the cellular levels of isoprenoid compounds. Additionally, it rendered cells hypersensitive to lovastatin, an inhibitor of 3-hydroxy-3-methylglutaryl coenzyme A reductase (HMGR) that acts upstream of FPP synthase in the isoprenoid pathway. While HMGR is encoded by two genes, *HMG1* and *HMG2*, only *HMG2* overexpression was able to restore growth of the *yta7Δ* cells in the presence of lovastatin. Moreover, the expression level of the *S. cerevisiae* *YTA7* gene was altered upon impairment of the isoprenoid pathway not only by lovastatin but also by zaragozic acid, an inhibitor of squalene synthase. Altogether, these results provide substantial evidence of Yta7p involvement in the regulation of isoprenoid biosynthesis.

Introduction

Regulation of the isoprenoid pathway has been extensively studied in mammals for many years (Goldstein & Brown, 1990). These studies were mainly motivated by the finding that steroid hormones and cholesterol are products of this pathway. In lower eukaryotes, such as yeasts, the isoprenoid pathway has also attracted considerable attention because the biosynthesis of ergosterol, which is the fungal equivalent of cholesterol, is the main target of the currently available antifungal drugs (Kauffman, 2006).

In eukaryotic cells, the first reaction considered as a rate-limiting step of isoprenoid pathway is the conversion of 3-hydroxy-3-methylglutaryl (HMG) coenzyme A into

mevalonate (Fig. 1). In yeast, this reaction is catalyzed by two isoforms of HMG coenzyme A reductase (HMGR) encoded by *HMG1* and *HMG2* (Basson *et al.*, 1986). *HMG1* is mainly expressed during exponential growth on glucose, whereas *HMG2* is preferentially expressed during the stationary phase and under anaerobiosis (Casey *et al.*, 1992). In humans, HMGR has been shown to control the biosynthesis of cholesterol (Balasubramaniam *et al.*, 1976), a critical health risk factor. Once mevalonate is synthesized, it is converted in subsequent reactions by the *ERG20*-encoded farnesyl diphosphate (FPP) synthase into FPP. The first of these reactions is the production of isopentenyl diphosphate (IPP), followed by the condensation of IPP with its isomer, dimethylallyl diphosphate (DMAPP), to form geranyl diphosphate (GPP). Then, FPP is produced by addition of

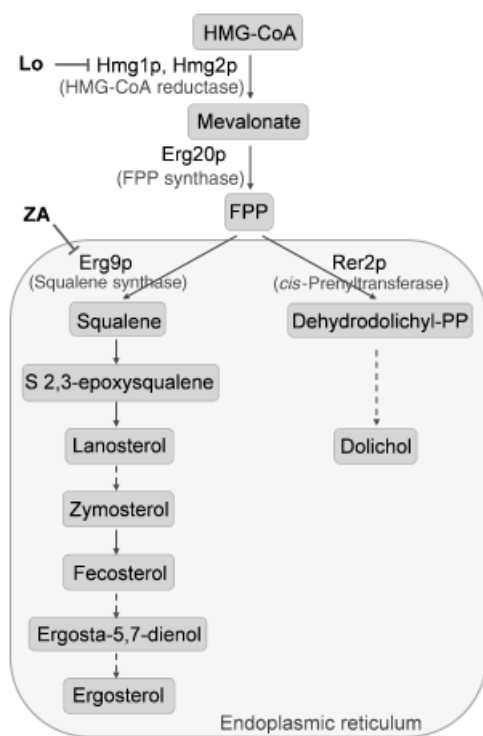


Fig. 1. An outline of the isoprenoid pathway in yeast. Dashed lines indicate multicomponent steps.

another molecule of IPP to GPP (Anderson *et al.*, 1989). FPP constitutes a branch-point of the pathway that serves as a substrate for enzymes that synthesize steroid and nonsteroid (i.e. dolichols, ubiquinones and heme) compounds as well as prenyl groups for post-translational modification of proteins.

While the regulation of HMGR is relatively well studied (for a review, see Panda & Devi, 2004), not much is known about the regulation of FPP synthase. In mammals, it has been shown that FPP synthase, similar to HMGR, is regulated at the transcriptional level in response to changing amounts of cellular sterols (Goldstein & Brown, 1990). More recently, oxysterol was identified as a source of signal for HMGR, both in mammals and in yeast (Gardner *et al.*, 2001). However, in the yeast, the function of sterol-derived signal was indirect, as it served to enhance the ability of a nonsterol (FPP)-derived signal to promote HMGR degradation.

In order to investigate the regulation of FPP synthase in yeast, we searched for protein partners of this enzyme using the two-hybrid method. We identified Yta7 protein as one of the FPP synthase partners and provide evidence for an involvement of this protein in the regulation of the isoprenoid pathway in yeast.

Table 1. Yeast strains, genotypes and plasmids used in this study

Strain name	Relevant genotype	Reference
BY4742	<i>MATα*</i>	Brachmann <i>et al.</i> (1998)
Y15922	<i>MATα*</i> <i>yta7::kanMX4</i>	Brachmann <i>et al.</i> (1998)
YDL401	<i>MATα⁺</i>	Lafontaine & Tollervey (1996)
K314	<i>MATα⁺ TAPtag-N-YTA7</i>	This study
Y115	<i>MATα*</i> pRS316	This study
Y113	<i>MATα*</i> <i>yta7::kanMX4</i> -pRS316-YTA7-ProtA	This study
Y114	<i>MATα*</i> <i>erg20::kanMX4</i> -pRS316-ERG20-ProtA	This study
Y110	<i>MATα*</i> -pNEV	This study
Y111	<i>MATα*</i> -pNEV-HMG1	This study
Y112	<i>MATα*</i> -pNEV-HMG2	This study
L40	<i>Mata his3D200 trp1-901 leu2-3112 ade2 LYS2::(4lexAop-HIS3) URA3::(8lexAop-LacZ)GAL4</i>	Invitrogen

*Background: *his3 Δ 1 leu2 Δ 0 lys2 Δ 0 ura3 Δ 0*.

[†]Background: *ura3-52 trp leu2 Δ 0 his3- Δ 200 gal2 gal108*.

Materials and methods

Yeast strains, culture media and drug treatment

The yeast strains used in this study are listed in Table 1. Transformation of yeast was performed according to the lithium acetate/single-stranded DNA/polyethylene glycol procedure (Gietz *et al.*, 1995). Unless otherwise stated, yeast strain BY4742 was cultivated in YPD (1% yeast extract, 1% Bacto peptone and 2% glucose) medium at 28 °C, and cells were collected at the exponential phase of growth (OD_{600 nm} 0.5). For enzymatic assays, yeast cultures were grown overnight in YPD medium; then they were diluted to OD_{600 nm} 0.1 and, where indicated, lovastatin was added at a final concentration of 25 μ g mL⁻¹. Cells were collected when cultures reached OD_{600 nm} 0.5. For cDNA microarray experiments, overnight cultures were diluted to OD_{600 nm} 0.05, grown to OD_{600 nm} 0.2 and then divided into three equal parts. One part served as a control, while the second and third parts were supplemented with lovastatin or zaragozic acid at the final concentration of 10 or 25 μ g mL⁻¹, respectively. After 10, 30 and 60 min, cells were collected by rapid centrifugation, frozen in liquid nitrogen and kept at -80 °C until use. For the lovastatin sensitivity test, serial 10-fold dilutions of exponentially growing cultures of each strain were spotted onto SD-Ura (0.7 g L⁻¹ yeast nitrogen base and 2% glucose, supplemented with adequate amino acids) agar plates containing 0 or 300 μ g mL⁻¹ of lovastatin. Lovastatin was purchased from A. G. Scientific Inc., and stock solution was prepared as described in Lorenz & Parks (1990). Zaragozic acid was kindly provided by Merck.

Yeast two-hybrid screen

The two-hybrid system was based on two plasmid-borne fusions that were cotransformed into the yeast host strain L40, which contained the inducible reporter genes *HIS3* and *LacZ* with a *lexA*-binding site in their promoters (Chien *et al.*, 1991). The *ERG20* gene was fused in frame to the 3' end of the DNA-binding domain from the LexA protein (pBTM116) (Vojtek *et al.*, 1993). The FRYL (I-1651) library based on the two-hybrid pACT2 plasmid was a generous gift from P. Legrain and M. Fromont (Institute Pasteur, Paris). Cells obtained after cotransformation of L40 strain with pBTM116-ERG20 'bait' plasmid and the FRYL library were plated onto selective media without tryptophan, leucine and histidine. β -Galactosidase activity was measured in the resulting clones using a filter assay (Cordier *et al.*, 1999). Plasmids containing positive clones fused to the *GAL4*-activating domain were isolated using the KC8 *Escherichia coli* strain (Clontech) and were used to retransform the L40 yeast strain separately or together with pBTM116-ERG20 (positive control) or pBTM116-HEM15 (negative control) for revalidation of growth on a histidine-deficient medium and β -galactosidase activity. After sequencing, the inserts of isolated clones were identified by searching the sequences from the *Saccharomyces* genome database (SGD).

Measurement of β -galactosidase activity

For a qualitative two-hybrid assay on plates, the β -galactosidase activity was measured using the rapid filter assay according to Cordier *et al.* (1999). The spectrophotometric method (Vojtek *et al.*, 1993) was then used to measure the β -galactosidase activity in cell-free extracts from positive clones that were cultivated in selective media and harvested at OD_{600 nm} 1.0. Extracts were prepared in 50 mM phosphate buffer and used as the enzyme source. The enzyme activity was calculated as nanomoles of *o*-nitrophenyl- β -D-galactopyranoside hydrolyzed per minute per milligram of protein.

Protein localization

By immunofluorescence microscopy

Strains Y113 and Y114 were fixed in the exponential phase of growth (OD_{600 nm} 0.5), with formaldehyde in buffer A (50 mM KPO₄, pH 6.5, 0.5 mM MgCl₂). Then, cells were washed with buffer B (buffer A supplemented with 1.2 M sorbitol) and cell walls were digested by a cocktail of zymolyase and glusulase. The protoplasts obtained were washed and incubated for 1 h in buffer F (0.7 mM KH₂PO₄, pH 7.4, 145 mM NaCl, 0.1% BSA and 150 mM NaN₃, pH 7.4). Incubations with the primary (overnight) and secondary antibodies (2 h, conjugated with Texas Red/Molecular Probes) were followed by extensive washing. Finally, proto-

plasts were resuspended in buffer B and spread on polylysine-coated slides (Sigma). The slides were allowed to dry and then a rapid (1 min) staining with 4',6'-diamino-2-phenylindole was performed, after which protoplasts were washed twice with water, covered with a mounting solution and by a cover glass. Subsequently, they were observed using a Nikon Eclipse E800 microscope (magnification $\times 100$, oil immersion).

By subcellular fractionation

Twenty OD units of yeast cells (strain Y113 and Y114) cultivated in YPD were collected at OD_{600 nm} 0.5 by centrifugation, washed once with water and incubated 45 min at 30 °C in buffer containing 100 mM Tris/HCl, pH 9.5, 100 mM β -mercaptoethanol, 10 mM NaN₃ and 40 mM EDTA. To obtain spheroplasts, cells were incubated 30 min at 30 °C in buffer containing 1.5 M sorbitol, 10 mM Tris/HCl, pH 7.5 and 10 U of zymolyase per 1 OD unit. Spheroplasts were washed (1 M sorbitol, 150 mM KAc, 5 mM MgAc and 20 mM HEPES, pH 6.8), resuspended in cold TEA buffer (10 mM triethanolamine, pH 7.5, 10 mM KF, 10 mM NaN₃, 1 mM EDTA and 0.8 M sorbitol) and were gently homogenized (Potter homogenizer). To separate organelles, after each round of the following centrifugations (500 g, 5 min; 3000 g, 15 min; 10 000 g, 30 min; 25 000 g, 30 min; 50 000 g, 60 min; and 100 000 g, 90 min), the pellet was kept aside and the supernatant obtained was submitted for the next centrifugations. To decrease the level of contamination between fractions, pellets were resuspended in TEA buffer and centrifuged again. The presence of Yta7-ProtA, Erg20-ProtA or Dpm1p in each fraction was verified using standard Western blot analysis and the secondary antibodies conjugated with alkaline phosphatase (Dako, Cytomation).

Membrane solubilization

Yeast strain K314 expressing tandem affinity purification (TAP)-tagged YTA7 was grown to OD_{600 nm} 0.5. A crude extract was prepared using glass beads in TEG buffer (50 mM Tris/HCl, pH 7.5, 1 mM EDTA and 5% v/v glycerol) supplemented with Protease Inhibitors Cocktail (Sigma). The cellular extract was divided into equal volumes, and to each part, one of the following reagents was added to a final concentration: 1 M NaCl, 0.1 M Na₂CO₃, pH 11.5, 2 M urea or 2% Triton X-100. Each sample was supplemented with 1 mM PMSE. After 1 h of incubation at 4 °C, samples were centrifuged for 1.5 h at 100 000 g. Proteins from the pellet and supernatant fractions were used for Western analysis. Yta7-TAP protein was visualized with primary antibodies developed in rabbit and secondary antibodies conjugated

with alkaline phosphatase (Dako, Cytomation), which was then detected with BCIP/NBT solution (Sigma).

Enzyme assays

Assay of FPP synthase

Yeast crude extract that served as the source of FPP synthase was prepared in 50 mM phosphate buffer, pH 7.5, supplemented with 5 mM iodoacetamide. The enzyme assay was carried out according to Chambon *et al.* (1991). The reaction mixture in the final volume of 100 μ L contained 50 mM phosphate buffer, pH 7.5, 1 mM MgCl_2 , 5 mM iodoacetamide, 1×10^5 c.p.m. [^{14}C]IPP (specific activity 52 Ci mol^{-1}), 60 μ M IPP, 120 μ M DMAPP and 100 μ g of total protein from the yeast extract. After 5 min at 37 °C, the reaction was stopped by addition of 0.5 mL of H_2O , 1 mL of hexane and 0.2 mL of 1 M HCl. After 30 min of additional incubation at 37 °C, samples were vigorously mixed; the upper phase was separated and washed with water. After removal of water, the remaining organic phase was mixed with five volumes of scintillation liquid and radioactivity was counted using an LKB Wallac 1209 RockBeta Counter.

Assay of *cis*-prenyltransferase

The activity of this enzyme was measured in the membrane fraction isolated as follows: cells were suspended in cold Tris solution I (50 mM Tris/HCl, pH 7.4, 15 mM MgCl_2 and 9 mM β -mercaptoethanol) and broken by vortexing with glass beads on ice. The supernatant obtained from the first centrifugation (4 °C, 5 min, 2000 g) was centrifuged again for 1.5 h, 50 000 g at 4 °C. The pellet was resuspended in Tris solution II (50 mM Tris/HCl, pH 7.4, 3.5 mM MgCl_2 and 6 mM β -mercaptoethanol), homogenized (Potter homogenizer). The homogenous membrane fraction was stored at –80 °C until use. The enzyme assay was carried out according to Szkopinska *et al.* (1997). In a final volume of 250 μ L, the reaction mixture contained 50 mM phosphate buffer, pH 7.5, 0.5 mM MgCl_2 , 20 mM β -mercaptoethanol, 10 mM KF, 3×10^5 c.p.m. [^{14}C]IPP (specific activity 52 Ci mol^{-1}), 46 nM FPP and the isolated yeast membranes (450 μ g of protein). The reaction was carried out for 1 h at 30 °C and was terminated by addition of a chloroform:methanol (3:2) mixture. Clear organic phase was concentrated under a stream of nitrogen and subjected to thin-layer chromatography on HPTLC RP-18 plates developed in 50 mM H_3PO_4 in acetone. Autoradiograms were obtained after 14 days of exposure against an X-ray film. Additionally, the zones containing radiolabeled polyprenols were scraped from the plates and the radioactivity was measured with a scintillation counter (LKB Wallac 1209 RockBeta Counter).

Measurement of sterol composition

Sterols were extracted from yeast cells grown in liquid YPD medium to OD 4 as described previously (Joets *et al.*, 1996). Sterols were quantified using UV absorption according to Jani *et al.* (1993), and the various species were analyzed by gas–liquid chromatography on a capillary RSL 150 column (Altech). The gas chromatograph (GC 6000; Carlo-Erba) was supplied with a flame-ionization detector and an on-column injector. Cholesterol was used as an internal standard to determine the relative retention times.

cDNA microarrays

cDNA microarrays were analyzed as described in Kuranda *et al.* (2006). Briefly, total RNA was extracted from frozen cells (10 units of OD_{600 nm}) using the RNeasy Mini kit (Qiagen). The quantity and the quality of the extracted RNA were determined by microcapillary electrophoresis (Bioanalyzer 2100; Agilent). To reduce biological and systematic variability, total RNA from four independent cultures was hybridized to four independent dendri-chips (Le *et al.*, 2003) including the dye swap technique. Hybridization was carried out in an automatic hybridization chamber (Discovery, Ventana Medical System Inc.) for 10 h at 42 °C. The hybridization signals were detected by a 4000B laser scanner (Axon Instruments) and transformed to numerical values using the GENEPIX software version 3.01. Raw intensities were corrected by the background, log transformed and normalized by the mean log intensity of all spots. Log ratios of normalized intensities from duplicate samples were tested for statistical significance using Student's *t*-test. Raw data were processed using homemade BIOPLOT/BIOCUST software and are accessible at <http://biopuce.insa-toulouse.fr/jmflab/cellwallgenomics/>. The differentially expressed genes (1.5-fold change in expression and *P*-value ≤ 0.05) were attributed to functional categories according to SLIM MAPPER (<http://yeastgenome.org/>), which currently contains 7292 genes annotated to at least one GO term. This work is fully MIAME-compliant (MIAME, Minimum Information About a Microarray Experiment) and has been deposited at the GEO website (accession number GSE 12984).

Results

The Yta7 protein interacts with Erg20p in the yeast two-hybrid screen

In order to find proteins that could potentially regulate FPP synthase, we searched for protein partners of this enzyme encoded by *ERG20* using a yeast two-hybrid system. To this end, *ERG20* was fused upstream of the sequence encoding the binding domain of LexA protein, and the expressed fusion protein was used as a bait to screen for interacting

Table 2. Proteins interacting with FPP synthase in two-hybrid screen

Gene name	No. of clones	β -Galactosidase activity (nmol min ⁻¹ mg ⁻¹ of protein)	Protein function
YTA7	3	13	Protein of unknown function; member of CDC48/PAS1/SEC18 family of ATPases
HIR3	1	27	Transcriptional corepressor involved in the cell cycle-regulated transcription of histone genes; involved in position-dependent gene silencing and nucleosome reassembly
HAP1	2	56	Zinc finger transcription factor which regulates gene expression in response to levels of heme and oxygen
SRB4	3	450	Subunit of RNA polymerase II mediator complex; essential for transcriptional regulation
ABC1	2	275	Protein required for ubiquinone (coenzyme Q) biosynthesis and for respiratory growth

proteins fused to the activating domain of Gal4p. The first screen was based on interactions indicated by a qualitative assay of β -galactosidase in positive clones. The plasmids were isolated from these clones, and the genes encoding the interacting proteins were identified by sequencing. We have identified five proteins that could potentially interact with FPP synthase (Table 2). Two of them, Abc1 and Hap1 proteins, had previously been shown to be functionally linked to the isoprenoid pathway. This could be taken as an indication of the high quality of the yeast two-hybrid library. Abc1p is required for ubiquinone Q biosynthesis (Do *et al.*, 2001; Tauche *et al.*, 2008), whereas Hap1/Elp4p is part of the six-subunit Elongator complex, which is a major histone acetyltransferase component of the RNA polymerase II holoenzyme responsible for transcriptional elongation and participates in the transcriptional regulation of genes involved in ergosterol biosynthesis (Hickman & Winston, 2007). Altogether, although the above-described proteins are functionally linked to the isoprenoid biosynthesis, their observed interaction with Erg20p could be nonphysiological. Two other proteins, Hir1 and Srb4, have been reported to be involved in general transcriptional regulation (Kang *et al.*, 2001; Prochasson *et al.*, 2005), but their linkage to the isoprenoid pathway is not known. The last partner was Yta7, a protein whose function has not yet been well characterized. Despite showing the weakest interaction among the partners identified by the β -galactosidase activity, Yta7p was isolated in three independent clones.

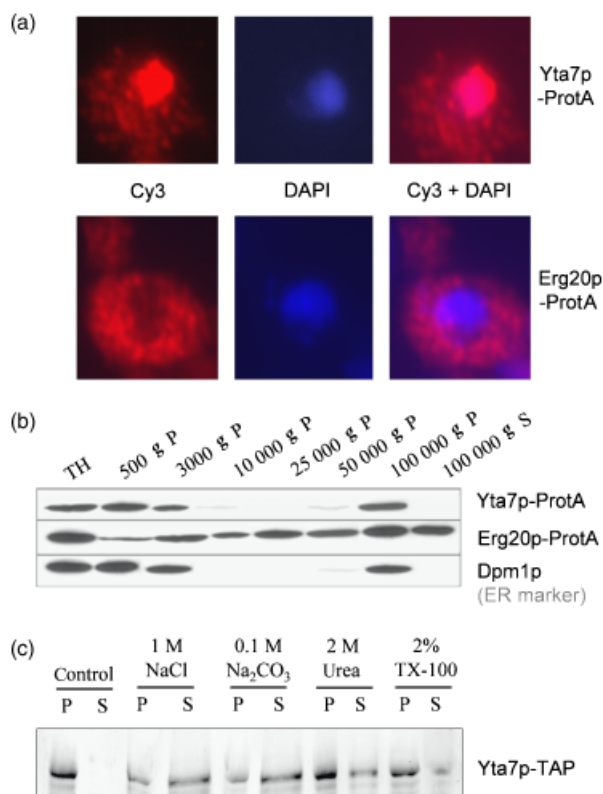


Fig. 2. Localization of Yta7p and its binding to cellular membranes. (a) ProtA-Erg20p or ProtA-Yta7p was detected with antibody conjugated with Cy3 fluorophore. After 4',6'-diamino-2-phenylindole staining of DNA, cells were subjected to immunofluorescence microscopy (magnification $\times 100$, oil immersion). (b) Yeast spheroplasts from cells expressing Erg20p-ProtA or Yta7p-ProtA were homogenized and subjected to a series of centrifugations. Aliquots were taken from pellets (P) and the supernatant (S) and analyzed by Western blot. Dpm1p was used as an ER-membrane marker. (c) Membrane fraction isolated from the yeast strain carrying the Yta7-TAP protein was treated with the indicated compounds. After centrifugation (100 000 g), the efficiency of Yta7 protein solubilization was verified by Western blotting.

Yta7 is a peripheral membrane protein located in the nucleus and in the endoplasmic reticulum (ER)

In a large-scale study, Yta7p has been localized to the nucleus (Huh *et al.*, 2003), while another group suggested retention of this protein at the ER membrane due to the presence of specific signals at both the N- and the C-termini of this protein (Teasdale & Jackson, 1996). Because most of the enzymatic steps in the isoprenoid pathway take place in the ER compartment, we decided to re-examine the cellular localization of this protein as well as that of Erg20p. To this end, the two proteins were fused to the ProtA tag and their localization was visualized by immunofluorescence microscopy using an immunoglobulin G fused to the Cy-3 fluorophore. As shown in Fig. 2a, the Yta7-ProtA fusion

protein was detected in the nucleus and as scattered dots in the cytoplasm, while Erg20p-ProtA was distributed in the cytoplasm, but not in the nucleus. Because these results were inconclusive regarding the potential colocalization of the two proteins in the ER, we analyzed their localization by subcellular fractionation (Fig. 2b). We found that Erg20p was a ubiquitous protein with the highest abundance in the 100 000 g pellet containing the ER compartment. Yta7p was present in the 500 g, 3000 g and 100 000 g pellet fractions, but not in the soluble 100 000 g fraction, which excluded its free presence in the cytoplasm. This distribution pattern was identical to that of Dmp1p (dolichyl phosphate mannose synthase), known to be tightly associated with the ER compartment (Preuss *et al.*, 1991).

As inferred from the protein sequence, Yta7p could be a transmembrane protein. Therefore, we examined the nature of its association with the cellular membranes by the conventional membrane solubilization technique. Membrane fractions obtained from exponentially growing yeast cells expressing the Yta7-TAP fusion protein were treated with various polar reagents or a detergent known to release transmembrane proteins from the membrane. All reagents tested were able to release Yta7p from cellular membranes (Fig. 2c), whereas Dpm1 used as a control was released only after treatment with Triton X-100 in accordance with its transmembrane localization (data not shown). Altogether, these results indicated that Yta7p is not a transmembrane protein, but it associates weakly with the membrane, and that both Yta7p and Erg20p occupy the same cellular compartment, which makes their putative interaction feasible.

Effects of *YTA7* deletion on enzymes and metabolites of the isoprenoid pathway

Because our fractionation procedure indicated a likely colocalization of Yta7p with a fraction of the Erg20 protein, and these two proteins were found to potentially interact with each other, we investigated whether this interaction could alter the activity of the FPP synthase. However, neither the Erg20 protein stability (protein synthesis was blocked with cycloheximide and the time needed for Erg20p degradation was measured, results not shown) nor the FPP synthase activity was modified in the cells lacking *YTA7* (Table 3). Next, we checked the activity of *cis*-prenyltrans-

ferase that utilizes FPP as a substrate and regulates the branch of the isoprenoid pathway devoted to dolichol biosynthesis. In the $\Delta yta7$ mutant, the activity of *cis*-prenyltransferase increased by 18% ($P < 0.05$, three assays in duplicate). This decrease was more pronounced when this yeast mutant was cultivated with a low dose of the HMGR inhibitor lovastatin (Alberts *et al.*, 1980) ($25 \mu\text{g mL}^{-1}$), and reached 30%.

Next we verified whether the deletion of *YTA7* affected another branch of the isoprenoid pathway devoted to sterol biosynthesis. Using liquid–gas chromatography to measure several sterol compounds, we found that the level of squalene increased twofold and lanosterol was 30% higher in the $yta7\Delta$ mutant as compared with the wild-type strain. The levels of sterols downstream of lanosterol, such as

Table 3. Enzymatic activity of *cis*-prenyltransferase (PT) and FPP synthase in $yta7\Delta$ mutant

Strain	<i>cis</i> -PT (c.p.m. min ⁻¹ mg ⁻¹ of protein)		FPP synthase (c.p.m. min ⁻¹ mg ⁻¹ of protein)	
	Control	+Lo	Control	+Lo
wt	164 ± 6	174 ± 10	20 600 ± 520	18 400 ± 650
$yta7\Delta$	195 ± 14	229 ± 11	20 400 ± 550	20 900 ± 500

wt, wild type.

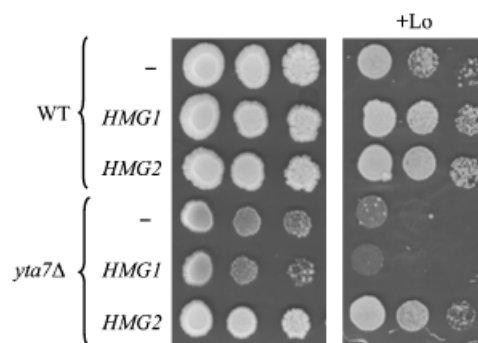


Fig. 3. *HMG2*, but not *HMG1*, is a multicopy suppressor of lovastatin sensitivity of the $yta7\Delta$ mutant. Serial dilutions of exponentially growing cultures were spotted onto SD agar plates containing 0 or $300 \mu\text{g mL}^{-1}$ lovastatin (Lo). Growth was scored after 5 days of incubation at 28°C . *HMG1*, strain bearing pNEV*HMG1*; *HMG2*, strain bearing pNEV*HMG2*; –, strain bearing empty vector; wt, wild type.

Table 4. Sterol compounds in the $yta7\Delta$ mutant

Strain	Squalene ($\mu\text{g g}^{-1}$ dry weight)	Lanosterol ($\mu\text{g g}^{-1}$ dry weight)	Zymosterol ($\mu\text{g g}^{-1}$ dry weight)	Fecosterol ($\mu\text{g g}^{-1}$ dry weight)	Ergosta-5,7-dienol ($\mu\text{g g}^{-1}$ dry weight)	Ergosterol ($\mu\text{g g}^{-1}$ dry weight)
wt	56 ± 4	749 ± 53	470 ± 33	543 ± 38	1098 ± 77	3112 ± 217
$yta7\Delta$	114 ± 9	937 ± 70	451 ± 34	512 ± 39	1000 ± 75	3045 ± 228

wt, wild type.

zymosterol, fecosterol, ergosta-5,7-dienol and the end product, ergosterol, did not change significantly (Table 4).

***HMG2* is a multicopy suppressor of lovastatin sensitivity of the *yta7Δ* mutant**

YTA7 is not essential and its deletion has not been reported to cause any severe phenotype, except a slight sensitivity to 6% ethanol (Agostoni Carbone *et al.*, 1998) and a decreased sensitivity to killer toxin 1 (Page *et al.*, 2003). In this work, we found that deletion of this gene slightly impaired the growth on SD medium and rendered cells hypersensitive to lovastatin (Fig. 3). We propose that the latter might be related to the reduced growth of the *YTA7* disruptant resulting from the reduced flux in the isoprenoid pathway.

On the other hand, the sensitivity of the *yta7Δ* mutant to zaragozic acid, an inhibitor of squalene synthase (Bergstrom *et al.*, 1993), was not different from that of the wild type (result not shown). The latter result might be linked to the fact that the *yta7Δ* mutant had an increased level of squalene. As lovastatin inhibits the activity of HMGR, we examined whether overexpression of either of the two genes encoding Hmg1p and Hmg2p could influence the *yta7Δ* phenotype. It turned out that the growth of the *yta7Δ* mutant on the selective medium (SD-Ura) as well as its sensitivity to lovastatin was restored to that of the wild type upon overexpression of *HMG2*, but not of *HMG1* (Fig. 3). Taken together, these results support the notion that Yta7 protein is involved in the performance of the isoprenoid pathway and that it might exert its function in co-operation with the *HMG1*-encoded HMGR.

***YTA7* expression level changes in cells treated with inhibitors of the isoprenoid pathway**

The results on Yta7p obtained so far suggested that this protein could alter the correct functioning of the isoprenoid pathway. Thus, we decided to verify whether, conversely, alteration of the isoprenoid pathway could have an impact on the expression of *YTA7*. Lovastatin and zaragozic acid are two drugs known to exert a repressing effect on the sterol branch of the isoprenoid pathway, but their action differs at the level of FPP biosynthesis. While lovastatin decreases FPP availability by inhibiting HMGR, zaragozic acid increases this level by blocking the squalene synthase encoded by *ERG9* (see Fig. 1) (Bergstrom *et al.*, 1993; Keller, 1996). In addition, to follow changes in *YTA7* expression, global changes at the transcriptome scale were monitored in response to lovastatin and zaragozic acid. These experiments were carried out in the presence of 25 µg mL⁻¹ lovastatin or 10 µg mL⁻¹ zaragozic acid, because these concentrations did not inhibit significantly the growth rate of the cells (results not shown). Using these low doses reduced the risk of

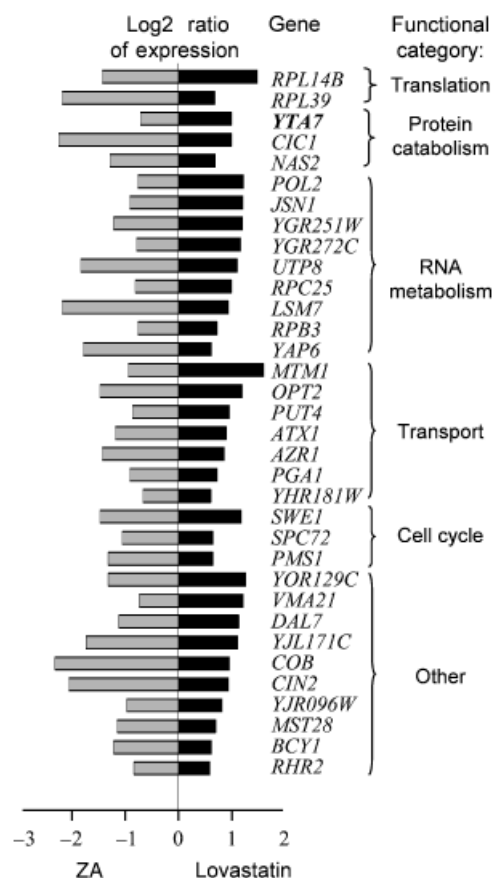


Fig. 4. Genes whose expression was increased by lovastatin (Lo) and repressed by zaragozic acid (ZA) treatment. Distribution of genes among functional categories was carried out according to the SGD database.

observing secondary effects due to growth inhibition and cell death.

Changes in gene expression upon addition of lovastatin to exponential yeast cells were relatively weak, but progressive, because expression of only 65 genes changed significantly after 10 and 30 min of the treatment, but this number increased to 272 genes after 60 min. In contrast, the effect of zaragozic acid was more marked. After 10 min, there were already 229 differentially expressed genes and this number remained almost unchanged after 30 and 60 min. In the case of both drugs, the majority of the differentially expressed genes fell into the broad functional categories of transport and organelle organization. These results will be described in detail elsewhere. In this work, we were especially interested in genes whose expression would be oppositely regulated in the presence of lovastatin and zaragozic acid, because such genes could be affected by a variation in the FPP level and thus related to the isoprenoid pathway. An analysis of the transcriptome data showed that 34 genes were found to behave in this manner: they all were upregulated by lovastatin, and downregulated by zaragozic acid.

(Fig. 4). These genes belonged to the functional categories of RNA metabolism, transcription, protein catabolism and biosynthesis, but none of them was directly linked to the isoprenoid pathway, except *YTA7*, which showed the expected trends, namely a reduced expression in response to zaragozic acid and an upregulation in response to lovastatin. In addition, this list of genes contained *OPT2* and *AZRI*, whose expression in response to these drugs was affected similar to as that of *YTA7*. Because these two genes were shown to be involved in resistance to azoles (Tenreiro *et al.*, 2000; Barker *et al.*, 2003), it was therefore possible that their upregulation in the presence of lovastatin was triggered as a protective mechanism against this fungicidal molecule.

In addition, it is worth noting that the expression of genes coding for Abc1 and Hir1 proteins, which were found to be FPP synthase partners in our two-hybrid system, was also affected by the inhibitors tested. *ABC1* was overexpressed upon treatment with lovastatin and *HIR3* with zaragozic acid (results not shown).

Discussion

In this work, we used the two-hybrid system to identify protein partners of the FPP synthase encoded by *ERG20*. This screen has led to the identification of five potential Erg20 interactors. Among them, protein encoded by *YTA7* was the only one predicted to be localized to the ER compartment, which is the major site of isoprenoid pathway reactions. We confirmed the ER localization of Yta7p and found that it is likely a peripheral membrane protein. According to the prediction of the hydrophobicity pattern of this protein, two transmembrane regions were possible (533–550 and 838–857 aa). However, a careful analysis showed that these hydrophobic regions lie within putative ATP-binding domains and they are likely buried in the protein's core (Beyer, 1997).

While this work was in progress, Tackett *et al.* (2005) proposed that Yta7p was a component of a Dpb4 chromatin-associated complex of proteins that helps to define and preserve boundaries separating silent and active chromatin. A function of Yta7p in this process was further supported by another work (Jambunathan *et al.*, 2005). In their study, *YTA7* was identified as a gene that, when mutated, allowed inappropriate spreading of silencing from the silent mating-type locus, HMR. Therefore, we considered the possibility that Yta7p could regulate the isoprenoid pathway through gene silencing and we verified whether genes encoding proteins involved in the isoprenoid pathway were located in the close vicinity of the Dbp4p complex on the chromosome. According to Tackett *et al.* (2005), there were 368 regions enriched in the Dbp4p complex in the yeast genome. Of all known isoprenoid-related genes, only *HMG1* lies in

the proximity of such regions (chromosome XIII, coordinates 118 898–120 089 bp) and could be potentially regulated by gene silencing. Importantly, as this effect could take place in the *yta7Δ* mutant, it could not occur in our experimental design because the multicopy plasmids we used bear only ORFs of the *HMG1* or *HMG2* genes, without the surrounding regions where the Dpb4p complex could potentially bind DNA. Nevertheless, while our data do not support an involvement of Yta7p in isoprenoid biosynthesis at the level of gene silencing, they do not exclude it.

While the involvement of Yta7p in gene silencing is consistent with its nuclear localization, we demonstrated that a significant amount of Yta7p is also present in the ER, where several enzymes of the isoprenoid pathway are located. However, localization of Yta7p to lipid particles, housing some of the proteins involved in the sterol biosynthetic pathway (Athenstaedt *et al.*, 1999; Mullner *et al.*, 2004), cannot be excluded, although it seems less likely because the computer analysis of the Yta7p structure reveals the presence of ER retention signals at the N and C termini of the protein (M. Greenberg, unpublished data). Moreover, under our experimental conditions, Yta7p colocalizes with Dpm1p (dolichyl phosphate mannosyl synthase), a protein marker for the ER membranes in yeast.

When the *YTA7* gene was sequenced, the resulting protein was attributed to the family of ATPases associated with diverse cellular activities because of its two predicted ATP-binding sites (Agostoni Carbone *et al.*, 1998). With increasing knowledge about this family, it became clear that the 'diverse cellular activities' were in fact mediated by unfolding and disassembly of proteins and protein complexes (for a review, see Lupas & Martin, 2002).

In accordance with this and previous work on Yta7p, we propose that this protein could play different roles in the cell depending on its cellular localization. This work provides evidence that one of its roles is the regulation of the isoprenoid pathway as indicated by the altered activity of *cis*-prenyltransferase as well as the cellular levels of some sterol compounds in the *yta7Δ* mutant. The accumulation of these compounds could be explained in two ways. Firstly, deletion of *YTA7* could stimulate the activity of enzymes synthesizing squalene (Erg9p) and lanosterol (Erg7p). Secondly, *YTA7* deletion could reduce the activity of enzymes utilizing these compounds (Erg1p and Erg11p, respectively). If the first explanation is true, it would also imply an additional negative regulation of the downstream steps of the sterol pathway, which were slightly inhibited in the *yta7Δ* cells. The existence of such a regulation has already been proposed, as the overexpression of the catalytic domain of *HMG1* results in squalene accumulation, but not in changes in the ergosterol level (Donald *et al.*, 1997). Altogether, changes in the isoprenoid composition and the fact that the expression of *YTA7* is dependent on the performance of the

isoprenoid pathway suggest that Yta7p could be a part of the mechanism regulating the isoprenoid pathway.

Another feature of the *yta7Δ* mutant supporting its involvement in the isoprenoid pathway was the fact that overexpression of *HMG2*, but not of *HMG1*, ameliorated the growth of the *yta7Δ* mutant on synthetic medium and in the presence of lovastatin. It is worth noting that under the standard growth conditions the protein encoded by *HMG2* provides only 20% of the total cellular activity of HMGR (Basson *et al.*, 1986). The *HMG1*-encoded HMGR is the dominant enzyme in the logarithmic phase of growth under aerobic conditions, while the *HMG2*-encoded enzyme becomes more important in anaerobiosis or in the stationary phase of growth. Therefore, it was proposed that Hmg1p would rather supply isoprenoids for processes requiring oxygen, such as heme biosynthesis, epoxidation of squalene and demethylation of lanosterol, while the need for Hmg2p activation would occur due to the accumulation of isoprenoids during anaerobiosis (Thorsness *et al.*, 1989; Casey *et al.*, 1992). As our results suggested that *HMG1* was not fully active in the *yta7Δ* cells, we verified whether the growth of these cells could be improved by addition of heme to the growth medium; however, no significant changes were observed (result not shown). At least two explanations are possible for the results obtained. Yta7p might be involved in the maintenance of Hmg1 stability. Consequently, in the *yta7Δ* strain, HMGR activity would become growth-limiting because of the decreased stability of Hmg1. Hmg2 cannot compensate because its gene is not induced because the cells are not in anaerobiosis. Upon addition of lovastatin, this phenotype (HMGR limiting) is exacerbated and leads to hypersensitivity. This is corrected only by the episomal copy of *HMG2*, which is not regulated by its own anaerobiosis-responding promoter. However, one could also envisage the possibility that overexpressed *HMG1* in the *yta7Δ* cells was fully active, but Yta7p was required further at the level of FPP-consuming enzymes. This possibility seems to be supported by the experiment showing a lack of an effect of *YTA7* deletion on the enzymatic activity of FPP synthase. Nevertheless, the different effects of *HMG1* and *HMG2* overexpression in the *yta7Δ* mutant in terms of lovastatin sensitivity additionally support the distinct regulation of these two genes. Altogether, the data presented strongly support the involvement of *YTA7* in the isoprenoid pathway.

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