

# Characterizing Sterol Defect Suppressors Uncovers a Novel Transcriptional Signaling Pathway Regulating Zymosterol Biosynthesis\*

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Melody Germann<sup>†1</sup>, Christina Gallo<sup>†1</sup>, Timothy Donahue<sup>‡</sup>, Reza Shirzadi<sup>‡</sup>, Joseph Stukey<sup>§</sup>, Silvia Lang<sup>¶</sup>, Christoph Ruckenstuhl<sup>¶</sup>, Simonetta Oliaro-Bosso<sup>||</sup>, Virginia McDonough<sup>§</sup>, Friederike Turnowsky<sup>¶</sup>, Gianni Balliano<sup>||</sup>, and Joseph T. Nickels, Jr.<sup>‡2</sup>

From the <sup>†</sup>Department of Biochemistry and Molecular Biology, Drexel University College of Medicine, Philadelphia, Pennsylvania 19102, the <sup>§</sup>Department of Biology, Hope College, Holland, Michigan 49422, the <sup>¶</sup>Institute of Molecular Biology, Biochemistry and Microbiology, Karl-Franzens-University Graz, A-8010 Graz, Austria, and the <sup>||</sup>Dipartimento di Scienza e Tecnologia del Farmaco, Facoltà di Farmacia, Università degli Studi di Torino, Corso Raffaello 31, I-10125, Turin, Italy

*erg26-1<sup>ts</sup>* cells harbor defects in the 4 $\alpha$ -carboxysterol-C3 dehydrogenase activity necessary for conversion of 4,4-dimethylzymosterol to zymosterol. Mutant cells accumulate toxic 4-carboxysterols and are inviable at high temperature. A genetic screen aimed at cloning recessive mutations remediating the temperature sensitive growth defect has resulted in the isolation of four complementation groups, *ets1-4* (*erg26-1<sup>ts</sup>* temperature sensitive suppressor). We describe the characterization of *ets1-1* and *ets2-1*. Gas chromatography/mass spectrometry analyses demonstrate that *erg26-1<sup>ts</sup>* *ets1-1* and *erg26-1<sup>ts</sup>* *ets2-1* cells do not accumulate 4-carboxysterols, rather these cells have increased levels of squalene and squalene epoxide, respectively. *ets1-1* and *ets2-1* cells accumulate these same sterol intermediates. Chromosomal integration of *ERG1* and *ERG7* at their loci in *erg26-1<sup>ts</sup>* *ets1-1* and *erg26-1<sup>ts</sup>* *ets2-1* mutants, respectively, results in the loss of accumulation of squalene and squalene epoxide, re-accumulation of 4-carboxysterols and cell inviability at high temperature. Enzymatic assays demonstrate that mutants harboring the *ets1-1* allele have decreased squalene epoxidase activity, while those containing the *ets2-1* allele show weakened oxidosqualene cyclase activity. Thus, *ETS1* and *ETS2* are allelic to *ERG1* and *ERG7*, respectively. We have mapped mutations within the *erg1-1/ets1-1* (G247D) and *erg7-1/ets2-1* (D530N, V615E) alleles that suppress the inviability of *erg26-1<sup>ts</sup>* at high temperature, and cause accumulation of sterol intermediates and decreased enzymatic activities. Finally using *erg1-1* and *erg7-1* mutant strains, we demonstrate that the expression of the *ERG25/26/27* genes required for zymosterol biosynthesis are coordinately transcriptionally regulated, along with *ERG1* and *ERG7*, in response to blocks in sterol biosynthesis. Transcriptional regulation requires the transcription factors, Upc2p and Ecm22p.

Sterols play crucial roles in the physiology of all eukaryotes. They are essential for membrane fluidity and function (1, 2) and have emerged as important second messenger lipids involved in proper developmental signaling (3, 4). The vertebrate sterol end product, cholesterol, can be synthesized *de novo* from acetyl coenzyme A through a multistep pathway (2), or obtained through the uptake of exogenous dietary chole-

sterol via the low density lipoprotein receptor (5, 6). An exquisite transcriptional signaling pathway comprised of a specific promoter SRE<sup>3</sup> and various SREBP isoforms regulates mammalian sterol gene expression in response to changes in sterol levels, thus maintaining cholesterol homeostasis (7). HMG-CoA reductase is the rate-limiting enzyme in higher eukaryotic sterol biosynthesis (2). It is regulated both at the transcriptional and protein stability levels in response to changes in sterol levels. The protein is rapidly degraded by a proteasome-dependent mechanism when sterol levels are high (8, 9).

The sterol biosynthetic pathway in the budding yeast *Saccharomyces cerevisiae* is highly conserved with mammals (10, 11). The only difference is that ergosterol is synthesized as the end product sterol rather than cholesterol. All of the genes have been cloned that are required for the biosynthesis of yeast ergosterol (11–13). Additional regulators of sterol metabolism and transport have been isolated and characterized, such as Erg28p (14) and the putative lipid transporter, Arv1p (15, 16). Physiological characterization of various *erg* mutants has revealed roles for yeast sterols in endocytosis (17), lipid raft formation and function (18, 19), cation and amino acid uptake (19, 20), and cell cycle regulation (21).

Yeast sterols have the ability to regulate endogenous sterol biosynthesis, presumably by a transcriptional feedback mechanism. Genes regulated by sterol availability include those encoding HMG-CoA reductase (*HMG1*), squalene synthase (*ERG9*), sterol C-5 desaturase (*ERG3*), and acetoacetyl-CoA thiolase (*ERG10*) (10) (Fig. 1). The *ERG10* promoter contains an active SRE-like element that has seven of the eight nucleotides that are found in the core mammalian SRE-1 consensus sequence (22). In addition, the *ERG3* promoter contains a 22-bp region that is involved in the sterol regulation of transcription (23). Vik and Rine (24) have uncovered a 7-bp SRE that regulates *ERG2* (sterol C-8 isomerase) and *ERG3*, which is shared among several *ERG* genes, as well as other genes needed for lipid metabolism, including the *LCB1/2* serine palmitoyltransferase genes, which are required for the first step in sphingolipid biosynthesis (10). The Upc2p and Ecm22p transcription factors bind to this SRE and most likely regulate sterol responsive gene transcription (24). Thus, the paradigm of transcriptional feedback regulation by a sterol product extends to *S. cerevisiae*, making this single cell

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<sup>1</sup> Both authors contributed equally to this work.

<sup>3</sup> The abbreviations used are: SRE, sterol response element; SREBP, sterol response element-binding protein; HMG-CoA, 3-hydroxy-3-methylglutaryl coenzyme A; CPRG, chlorophenol red- $\beta$ -D-galactopyranoside; TLC, thin layer chromatography; *ERG*, ergosterol; WT, wild type; MES, 4-morpholineethanesulfonic acid; GC/MS, gas chromatography/mass spectrometry.

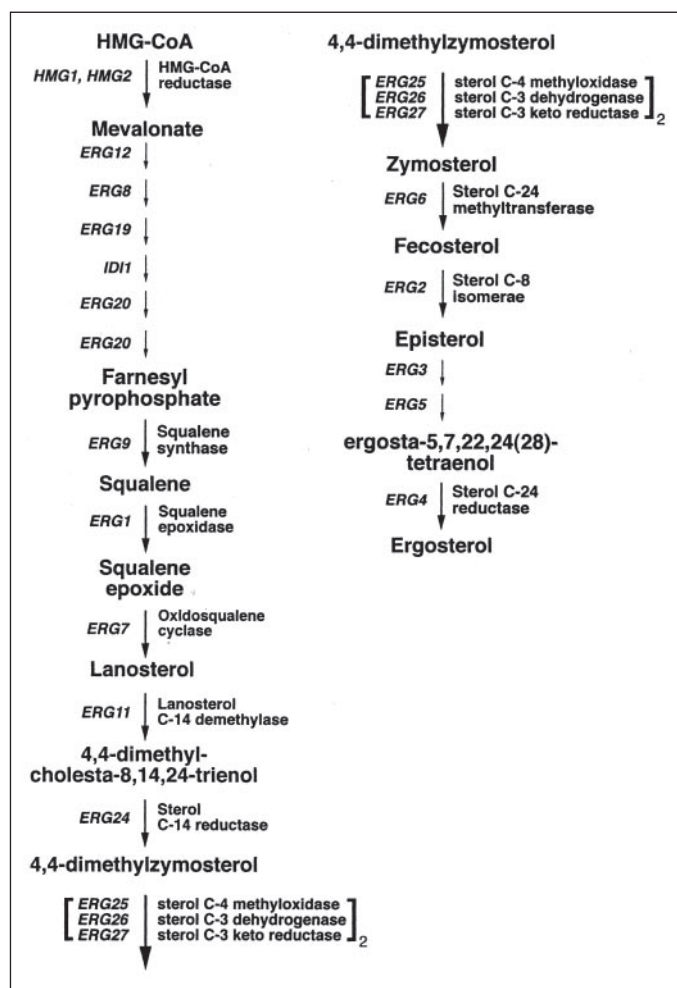


FIGURE 1. The sterol biosynthetic pathway in *S. cerevisiae*. The pathway at the point of the HMG-CoA reductase enzymatic step is presented.

eukaryote a highly useful tool in which to study the transcriptional regulation of sterol gene expression in eukaryotes.

We recently isolated a yeast strain, designated *erg26-1<sup>ts</sup>*, which harbors a *ts* allele of *ERG26* (25). Erg26p (4 $\alpha$ -carboxysterol-C3 dehydrogenase) (26), along with Erg25p (sterol-C-4 methyl oxidase) (27) and Erg27p (C-3 ketoreductase) (28), is required for the conversion of 4,4-dimethylzymosterol to zymosterol in the sterol biosynthetic pathway (2, 10). *erg26-1<sup>ts</sup>* cells accumulate 4-carboxysterols, but more importantly also harbor defects in LCB and complex sphingolipid metabolism (25), which suggests that sterol and sphingolipid biosyntheses may be coordinately regulated in yeast. Multiple studies in mammalian cell systems strongly suggest cross-talk regulation between sterol and sphingolipid pathways in higher eukaryotes (29–33). To further explore whether yeast have transcriptional and post-translational coordinate regulatory pathways governing sterol and sphingolipid homeostasis, we isolated and characterized recessive mutations that would rescue the inviability of *erg26-1<sup>ts</sup>* cells at high temperature. Four complementation groups were isolated, designated *ets1-4*. Our data demonstrate that *ets1-1* and *ets2-1* gene groups harbor recessive mutations in the *ERG1* squalene epoxidase and *ERG7* oxidosqualene cyclase genes, respectively. Using these mutant strains, we present evidence of a novel transcriptional signaling pathway, which coordinately regulates the transcription of several *ERG* genes at multiple points in the sterol pathway.

## EXPERIMENTAL PROCEDURES

**Strains, Media, and Miscellaneous Microbial Techniques**—The parental yeast strains used in this study are W303 (*MATa leu2-3,112 trp1-1 ura3-1 his3-11,15 can1-100*) or BY4741 (*MATa his3 leu2 met15 ura3*). Yeast strains were grown in either YEPD (1% yeast extract, 2% bacto-peptone, 2% glucose) or in synthetic minimal medium containing 0.67% Yeast Nitrogen Base (Difco) supplemented with the appropriate amino acids, adenine, and uracil. Antifungal drugs were either added directly to liquid YEPD medium or added to YEPD plates at the indicated concentrations. Lovastatin treatment was for 16 h, fluconazole, amphotericin B, and nystatin treatment was for 3 h. Yeast transformation was performed using the procedure described by Ito *et al.* (34). For routine propagation of plasmids, *Escherichia coli* XL1Blue cells were used and grown in LB medium supplemented with ampicillin (150  $\mu$ g/ml). Bacterial transformations were carried out by electroporation methods.

**Strain Construction and Plasmids**—Haploid strains harboring the *erg1::kan<sup>r</sup>*, *erg7::kan<sup>r</sup>*, *erg2::kan<sup>r</sup>*, *erg6::kan<sup>r</sup>*, and *erg24::kan<sup>r</sup>* deletion alleles were constructed using heterozygous null diploid strains obtained from the Research Genetics Deletion Collection and PCR. pRS416-*ERG1* and pRS416-*ERG7* were transformed into heterozygous diploids, and these cells were sporulated to obtain the necessary haploid strains. pRS416-*ERG1*, pRS416-*ERG7*, pRS416-*erg1-1*, and pRS416-*erg7-1* were constructed using the *URA3* containing centromeric plasmid, pRS416 (35). Yeast strains harboring individual deletions were verified by PCR analysis. All genomic sequences that were subcloned into vectors were obtained by PCR amplification using the high fidelity *pfu* polymerase. Mutations in *erg1-1* (G-A, nucleotide 740) and *erg7-1* (G-A and T-A, nucleotides 1588 and 1844, respectively) were determined by DNA sequencing of multiple overlapping *pfu* polymerase-generated fragments. The recombinant *erg1-1* and *erg7-1* alleles were constructed using the QuikChange<sup>®</sup> Multisite-directed Mutagenesis kit (Stratagene, La Jolla, CA) and were verified by DNA sequencing. All DNA sequences that were generated by PCR were sequenced and compared with the yeast genome data base. The yeast-integrating vector, YIp353, was used to construct all promoter-*LacZ* plasmids. YIp-*ERG1*, YIp-*ERG7*, YIp353-*ERG25*, YIp353-*ERG26*, and YIp353-*ERG27* were integrated at the *URA3* locus of each strain by digestion with *Stu*I. The following promoter sequences were used: *ERG1*, -650 to +2; *ERG7* -500 to +2; *ERG25* and *ERG27*, -1500 to +2; *ERG26*, -450 to +2.

**Isolation of *erg26-1<sup>ts</sup>* *ts* Suppressors**—To obtain recessive *ts* suppressors, *MATa* and *MAT $\alpha$*  haploid *erg26-1<sup>ts</sup>* cells were streaked onto prewarmed YEPD plates and grown for 3–6 days at 37 °C. 29 *MATa* and 30 *MATa ts* revertants were obtained. All individual *MATa* revertants were mated to each *MATa* and diploids were tested for growth at 27 °C and 37 °C. *MATa/MAT $\alpha$*  diploids that grew at 37 °C were considered homozygous for a single mutation suppressing the *ts* phenotype. Complementation groups were then assigned based on the results of the diploid growth assays. To obtain haploid strains harboring only the *erg1-1/ets1-1* or *erg7-1/ets2-1* allele, first a single haploid from each complementation group was selected, and the *erg26-1<sup>ts</sup>* allele was replaced with *erg26-1<sup>ts</sup>::erg26-1<sup>ts</sup>::URA3* in order to be able to track the *ts* allele through genetic crosses. Second, the resulting haploid strain was backcrossed to the opposite wild-type mating partner, the diploid was then induced to sporulate and haploid spores were selected based on their harboring the *erg26-1<sup>ts</sup>::erg26-1<sup>ts</sup>::URA3* allele and showing growth at 37 °C. This selection process was repeated five times. Spores harboring *erg1-1/ets1-1* or *erg7-1/ets2-1* alleles were then obtained from asc-containing tetraploid spores.

## Regulation of Zymosterol Biosynthesis

**Metabolic Radiolabeling and Analysis of Sterols and Neutral Lipids**—For steady state labeling of sterols, cultures were incubated at 30 °C with 5mCi/ml [<sup>14</sup>C]acetate (51 mCi/mmol) for 3 h. Radiolabeled sterols were extracted using chloroform, methanol (2:1) and analyzed by one-dimensional TLC using hexane, diethyl ether, acetic acid (80:20:2). Neutral lipid radiolabeling and TLC analysis using petroleum ether, diethyl ether, acetic acid (70:30:1) were performed as described previously (25). Sterol intermediates were visualized by autoradiography using Kodak XAR5 film.

**GC/MS Analysis of Sterols**—Sterols were extracted as part of the nonsaponifiable fraction essentially as described (36). Washed cells were resuspended in 1.5 ml of methanol, 1.0 ml of 60% potassium hydroxide, 1.0 ml 0.5% pyrogallol, and heated for 2 h at 85 °C. The nonsaponified material was extracted two times with petroleum ether, hexane (1:1). Cholesterol (20 μl of 0.5 mg/ml stock) was added to each sample for use as an internal standard. Samples were dried down under nitrogen and resuspended in 200 μl of a 1:1 (v/v) mixture of pyridine, bis(trimethylsilyl)trifluoroacetamide, 1% trimethylchlorosilane for trimethylsilylation (TMS) of free hydroxyl groups (37, 38). Each TMS treatment extract was diluted by addition of 0.5 ml of petroleum ether, hexane (1:1) and passed over a silica gel minicolumn that was pretreated with hexane, petroleum ether (1:1, v/v) (39). Sterols were eluted with hexane, ethyl acetate (1:1, v/v). The pooled flow-through and eluant fractions were dried down under nitrogen and resuspended in 200 μl of cyclohexane, dichloromethane (1:1, v/v). Sterol samples were analyzed by gas chromatography on a Hewlett-Packard model 6890 gas chromatograph using a 30-m HP-5 column. Standard instrument conditions were as follows: injection port temperature 250 °C, detector temperature 300 °C, initial oven temperature at 250 °C. After a 30-min hold at 250 °C, the oven temperature was increased at 2 °C/minute up to 280 °C, held for 15 min, and then increased at 5 °C/min to 310 °C and held for 5 min. Cellular sterol species were identified by comparison of peak position retention times to those of standard compounds (ergosterol, lanosterol, cholesterol (internal standard), and squalene) and/or relative to the cholesterol internal standard peak position (40–42) and by GC/MS analysis using data base files of sterol spectra. GC/MS analysis was performed on a Hewlett-Packard model 6890 gas chromatograph equipped with a HP-5MS capillary column and Hewlett-Packard model 5973 Mass Selective Detector. GC/MS run conditions were essentially the same as that described above for analysis of sterols by gas liquid chromatography.

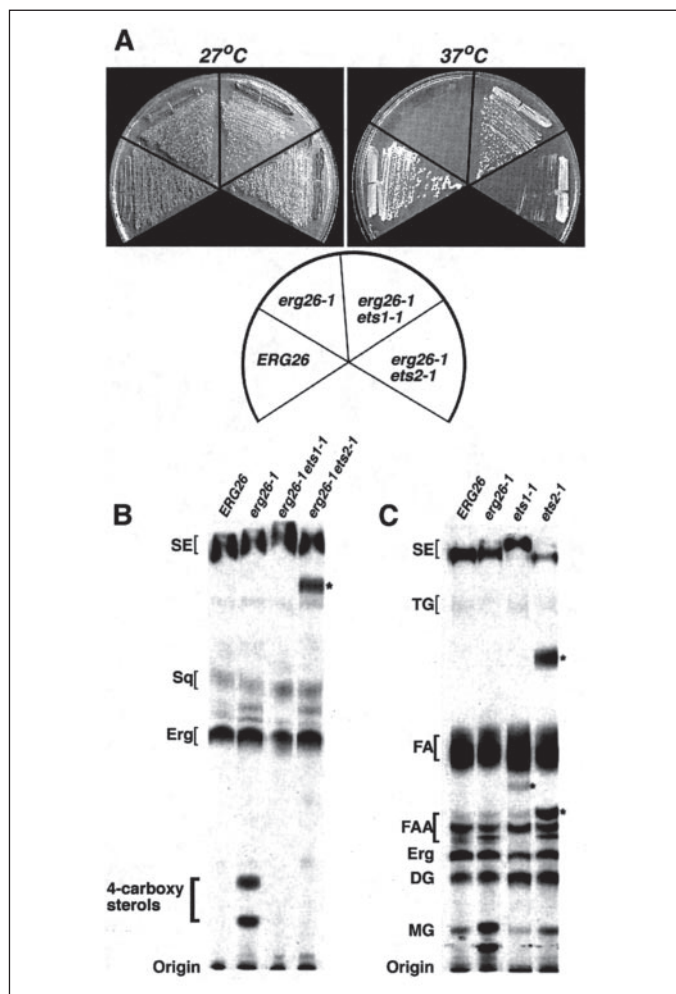
**Squalene Epoxidase Assays**—Yeast strains were grown aerobically in YEPD medium at 25 °C to late-log phase ( $A_{600}$  between 1.5 and 2). Cells were harvested, washed with 10 mM Tris-HCl, pH 7.4, and disrupted in a glass bead mill. Cell extracts were prepared by centrifugation at 12,000 × *g* for 20 min at 4 °C. Protein concentration was determined by the method of Bradford using bovine serum albumin as standard. 3 mg of total protein were incubated with the cofactor-substrate mix (5 mM NADPH, 10 mM sodium phosphate buffer, pH 7.4, containing 25 mM glucose 6-phosphate, 0.04 mM FAD, 2 units of glucose-6-phosphate dehydrogenase, 10 mM MgCl<sub>2</sub>, 5 mM mevalonic acid lactone, and 0.5 μCi of [<sup>14</sup>C]mevalonic acid) at 30 °C for 2 h. The enzymatic reaction was stopped by addition of 15% (v/v) KOH in methanol and incubation at 60 °C for 15 min. Lipids were extracted with hexane and analyzed by TLC on silica gel 60 plates using CHCl<sub>3</sub> as developing solvent. TLC plates were scanned in a PhosphorImager SI (Molecular Dynamics) and analyzed with Image Quant 5.1. Lipid species were identified by using respective standards on the same silica plates. Values of each lipid species represent percent of the total intensity/lane. The values represent the average of three independent experiments.

**[2-<sup>14</sup>C]Acetate Incorporation into Sterols for Oxidosqualene Cyclase Activity**—Sterol biosynthesis in whole yeast cells was followed by incorporation of [2-<sup>14</sup>C]acetate into nonsaponifiable lipids as previously described (43). Briefly, washed cells (2–4 × 10<sup>6</sup> cells) were resuspended in 1 ml of YPD, containing 0.1 mg/ml Tween 80, incubated with 0.2 μCi of [2-<sup>14</sup>C]acetate (50 mCi/mM) and shaken for 30 min or 3 h at 30 °C. Then, cells were harvested by centrifugation and saponified in 50% ethanol containing 15% KOH for 2 h at 80 °C. Nonsaponifiable lipids were extracted twice with 1 ml of light petroleum and separated on silica gel plates (Merck) using *n*-hexane/ethyl acetate (85:15, v/v) as developing solvent. Authentic standards of ergosterol, lanosterol, dioxidosqualene, oxidosqualene, and squalene were run with each sample. Radioactivity in separated bands was measured using a System Imaging Scanner (Packard).

**Oxidosqualene Cyclase Assays**—3S-2,3-[<sup>14</sup>C]Oxidosqualene was prepared by incubating a pig liver S<sub>10</sub> preparation with [<sup>14</sup>C]mevalonate as described (44), in the presence of 0.1 mM of the oxidosqualene cyclase (OSC) inhibitor U14266A (U14, 3β-(2-dimethylaminoethoxy)-androst-5-on-17-one) (45). After 3 h of incubation, the reaction was stopped by adding 1 volume of 15% KOH in methanol, and lipids were saponified at 80 °C for 30 min. The nonsaponifiable lipids were extracted three times with two volumes of light petroleum and separated by TLC on silica gel plates. The plates were first developed in light petroleum to a height of ~10 cm above the origin. After drying, the plates were developed to 15 cm above the origin with *n*-hexane/ethyl acetate (90:10, v/v). Radioactive bands corresponding to 2,3-oxidosqualene were scraped off and eluted with dichloromethane. The extract was brought to dryness under a stream of nitrogen and 3S-2,3-[<sup>14</sup>C]oxidosqualene was dissolved in benzene.

OSC activity in total yeast homogenate (6 mg of protein/ml) was determined as described elsewhere (43). Yeast cells were grown at 30 °C to early stationary phase in YPD medium. Cells were then pelleted at 3000 × *g* for 10, resuspended (0.15 g of wet cells/ml) in 0.02 M KH<sub>2</sub>PO<sub>4</sub> buffer, pH 7.4 containing 1.2 M sorbitol, and treated with lyticase (1 mg/g wet cells) for 60 min at 30 °C. The spheroplasts obtained after lysis were pelleted at 3000 × *g* for 10 min, washed twice in 0.02 M KH<sub>2</sub>PO<sub>4</sub> buffer, pH 7.4 containing 1.2 M sorbitol, and homogenized with a Potter device in 10 mM MES/Tris buffer, pH 6.9 containing 0.2 mM EDTA and 1 mM phenylmethylsulfonyl fluoride. 0.5 ml of the homogenate were incubated with 3S-2,3-[<sup>14</sup>C]oxidosqualene (5000 cpm, 25 μM) in the presence of Tween 80 (0.2 mg/ml) and Triton X-100 (1 mg/ml) in 10 mM MES/Tris, pH 6.9, and 0.2 mM EDTA for 30 min at 30 °C under vigorous shaking in a water bath. The reaction was stopped by adding 1 ml of 15% KOH in methanol, and lipids were saponified at 80 °C for 30 min. The nonsaponifiable lipids were extracted twice with 1 ml of light petroleum and separated on silica gel plates using dichloromethane as developing solvent. Radioactivity in 2,3-oxidosqualene and lanosterol was counted using a System Scanner (Packard). The amount of product formed was used for the calculation of the enzyme activity.

**Liquid β-Galactosidase Assays**—*LacZ* assays were performed as described in the Yeast Protocols Handbook (BD Biosciences Clontech) using the substrate, CPRG. Briefly, five independent colonies from a single strain were inoculated in minimal medium containing the appropriate amino acids and grown to exponential phase ( $A_{600}$  ~ 0.5–0.8). Cells were pelleted from 1.5 ml of the growing culture and resuspended in 300 μl of buffer 1 (100 mM HEPES, pH 7.3 containing 150 mM NaCl, 4.5 μM L-aspartate (hemimagnesium salt), 2.0 g of bovine serum albumin, and 100 μl of Tween 20). 100 μl of cells in buffer 1 were then subjected to three rounds of freeze/thawing using a dry ice/ethanol water bath. 700 μl of buffer 2 (20 ml of buffer 1 containing 27.1 mg of



**FIGURE 2. The *ets1-1* and *ets2-1* mutations suppress the *ts* and 4-carboxysterol accumulation phenotypes of *erg26-1<sup>ts</sup>* cells.** A, strains were streaked onto YEPD plates and grown at the indicated temperatures for 3 days. B, strains were grown in the presence of [<sup>14</sup>C]acetate for 3 h. Radiolabeled sterols were extracted and analyzed by TLC as described under "Experimental Procedures." C, neutral lipids were extracted and analyzed by TLC as described under "Experimental Procedures." SE, sterol esters; TG, triglycerides; FA, fatty acids; Sq, squalene; FAA, fatty acid alcohols; Erg, ergosterol; DG, diglycerides; MG, monoglycerides. asterisk, denotes uncharacterized [<sup>14</sup>C]acetate-radiolabeled neutral lipids.

CPRG) were added, and the reaction was allowed to proceed until color development became visible. The reaction was terminated by the addition of 500  $\mu$ l of 3.0 mM ZnCl<sub>2</sub>. Cellular debris was pelleted by centrifugation, and  $\beta$ -galactosidase activity was measured at A<sub>578</sub> using the resulting supernatant. Miller units were calculated as described (46). The Miller units represented are the average values of five independent experiments.

**mRNA Expression Analysis**—Cells were grown to exponential phase at 30 °C in YEPD. Total RNA was extracted from 3  $\times$  10<sup>7</sup> cells using glass beads, SDS and buffered-saturated hot phenol. Total RNA was denatured in formaldehyde-SSPE (10 mM NaH<sub>2</sub>PO<sub>4</sub> buffer, pH 7.7, containing 0.18 M NaCl and 1 mM EDTA) for 15 min at 65 °C. 20  $\mu$ g of denatured RNA was applied to a nylon membrane using a slot blot apparatus (Bio-Rad). Membranes were washed in SSPE, UV cross-linked and hybridized to gel-purified PCR products that were obtained using *pfu* polymerase. The hybridization buffer consisted of SSPE containing Denhardt's solution. Post-hybridization, membranes were washed several times with SSPE containing 0.1% SDS. Hybridization and all washes were performed at 65 °C. PCR products were [<sup>32</sup>P] radio-

labeled by random priming (Takara, Madison, WI). The level of *U2* mRNA expression was used as a loading control. *ERG* gene expression levels were determined by autoradiography (Kodak XAR5) followed by densitometry using a Bio-Rad model GS-670 Imaging Densitometer and Molecular Analyst software, version 1.4.1, or using a Storm 840 PhosphorImaging system and Storm Scanner Control software (Fig. 5E).

**Western Analysis of Erg1p Levels**—Strains were grown at 25 °C in YEPD medium to late-log phase and subsequently disrupted in a glass bead mill. Lysed cells were centrifuged at 12,000  $\times$  g for 20 min to obtain a total cell-free extract. 20  $\mu$ g of total cell extract protein from various *ERG* strains were resolved by SDS-PAGE and transferred onto a polyvinylidene difluoride membrane. Western blot analysis was performed using polyclonal anti-Erg1p antibodies and anti-Sui2p antibodies, respectively, and horseradish peroxidase-conjugated secondary antibodies as previously described (47). Erg1p migrates at 55 kDa and Sui2p, which serves as a loading standard, migrates at  $\sim$ 35 kDa.

## RESULTS

**Isolation of *ets* Suppressors**—We were interested in uncovering coordinate sterol/sphingolipid regulatory mechanisms in eukaryotes. Our approach was to isolate second site recessive mutations remediate the *ts* phenotype of *erg26-1<sup>ts</sup>* cells. We isolated four gene groups, *ets1-4* (*erg26-1<sup>ts</sup>* suppressors) (Fig. 2A, *ts* suppression by *ets1-1* and *ets2-1* are shown). The four groups fell into two separate categories, depending on their ability to eliminate the production of 4-carboxysterols in *erg26-1<sup>ts</sup>* cells (Fig. 2B, *erg26-1<sup>ts</sup>* versus *erg26-1<sup>ts</sup> ets1-1* and *erg26-1<sup>ts</sup> ets2-1*). *erg26-1<sup>ts</sup> ets1-1* and *erg26-1<sup>ts</sup> ets2-1* cells lacked the accumulation of these toxic intermediates (Fig. 2B), whereas 4-carboxysterols still accumulated in *erg26-1<sup>ts</sup> ets3-1* and *erg26-1<sup>ts</sup> ets4-1* (not shown). *erg26-1<sup>ts</sup> ets2-1* cells also accumulated a [<sup>14</sup>C]acetate-radiolabeled lipid not seen in other strains analyzed (Fig. 2B, asterisk). Based on these results, we characterized together the *ets1-1* and *ets2-1* mutant strains. Multiple genetic backcrosses were used to generate *ets1-1* and *ets2-1* haploid strains. The characterization of *ets3-1* and *ets4-1* will be discussed elsewhere.<sup>4</sup>

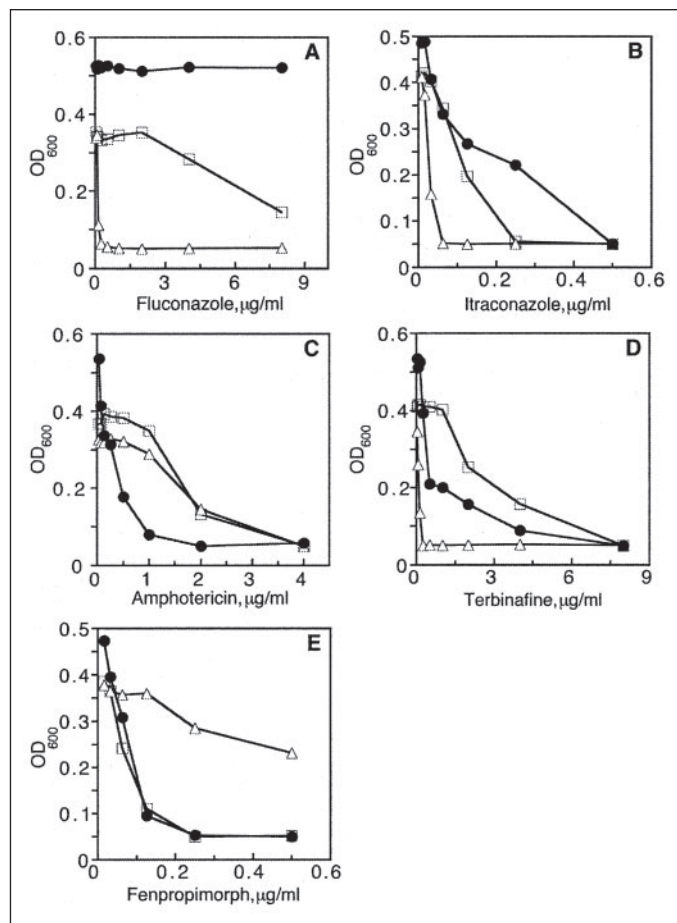
**Characterization of *ets* Suppressors**—Antifungal drugs, such as fluconazole and terbinafine, suppress the *ts* growth phenotype of *erg26-1<sup>ts</sup>* cells by decreasing the production of 4-carboxysterols; these target the Erg11p and Erg1p enzymes upstream of Erg26p, respectively (Fig. 1) (25). We reasoned that *ets1-1* and/or *ets2-1* cells might carry *erg* mutations upstream of *ERG26*, since these alleles cause the loss of 4-carboxysterol accumulation and remediate the *ts* phenotype of *erg26-1<sup>ts</sup>* cells (25). If so, they may show altered sensitivity to certain antifungal drugs targeting the sterol pathway. Many *erg* mutations have been isolated through drug resistance/sensitivity screens (10, 11).

We grew *ets1-1* and *ets2-1* cells in the absence and presence of various antifungal drugs and growth rate was determined over time. Both strains had an increased sensitivity to azole drugs (fluconazole and itraconazole) targeting the lanosterol C-14 demethylase (Fig. 1), Erg11p (Fig. 3, A and B), whereas they were resistant to the ergosterol binding agent, amphotericin (Fig. 3C). *ets1-1* cells also were sensitive to the allyamine, terbinafine (Fig. 3D), which targets the squalene epoxidase, Erg1p, but were resistant to the morpholine antifungal drug, fenpropimorph (Fig. 3E). *ets2-1* cells were mildly resistant to terbinafine (Fig. 3D), but showed normal sensitivity to fenpropimorph (Fig. 3E). Thus, *ets1-1* and *ets2-1* cells had antifungal drug resistance profiles that were different than wild-type cells.

*erg* mutations may also cause the accumulation of specific sterol intermediates, so we analyzed sterol and neutral lipid compositions of

<sup>4</sup> M. Germann, C. Gallo, and J. T. Nickels, Jr., manuscript in preparation.

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**FIGURE 3. *ets1-1* and *ets2-1* cells show differential sensitivities to various sterol biosynthetic inhibitors.** Cells were grown in synthetic medium at 30 °C for 48 h in the absence and presence of the indicated concentrations of the indicated sterol biosynthetic inhibitors (A–E). Cell growth was measured by determining the absorbance at  $A_{600}$  after 48 h. Wild type, closed circles; *ets1-1*, open diamonds; *ets2-1*, open boxes.

*ets1-1* and *ets2-1* cells. Using GC/MS, we observed that *erg26-1<sup>ts</sup> ets1-1* and *ets1-1* cells accumulated squalene, whereas they contained lower levels of most other sterol intermediates and the sterol end product, ergosterol (TABLE ONE). *erg26-1<sup>ts</sup> ets2-1* and *ets2-1* cells accumulated squalene epoxide and also contained much lower levels of ergosterol than wild-type cells. Neutral lipid composition demonstrated that *ets2-1* cells synthesize less sterol esters than wild type, whereas the sterol esters produced by *ets1-1* cells migrated differently than those seen in either wild-type or *erg26-1<sup>ts</sup>* cells (Fig. 2C). *ets2-1* also accumulated two lipid species not seen in wild type, one of which migrated similar to fatty acid alcohols (Fig. 2C, asterisk, *ets2-1*). *ets1-1* cells accumulated a minor species that migrated similar to fatty acids (Fig. 2C, asterisk, *ets1-1*). The exact molecular species of these lipids is not known. Thus, *ets1-1* and *ets2-1* cells harbor defects in sterol metabolism.

*ets1-1* and *ets2-1* cells may accumulate squalene and squalene epoxide, respectively, because they harbor recessive mutations in the squalene epoxidase, Erg1p, and oxidosqualene cyclase, Erg7p. To test this, we assayed Erg1p or Erg7p enzyme activities in wild-type and mutant cells. For squalene epoxidase activity, we determined the percent conversion of [ $^{14}$ C]mevalonic acid to squalene, squalene epoxide, lanosterol, and ergosterol in cell extracts (TABLE TWO). For oxidosqualene cyclase, we determined the conversion of [ $^{14}$ C]acetate to squalene, oxidosqualene, dioxidosqualene, and lanosterol *in vivo* (TABLE THREE), and we directly assayed for oxidosqualene cyclase activity using [ $^{14}$ C]oxidosqualene as a substrate.

Cell extracts from *ets1-1* cells accumulated radiolabeled squalene, the substrate for the Erg1p reaction, and were much less efficient at converting this sterol intermediate to the product of the reaction, squalene epoxide (TABLE TWO, W303, WT *versus* *erg26-1<sup>ts</sup> ets1-1* and *ets1-1*). *erg26-1<sup>ts</sup>* cell extracts also had a reduced ability to catalyze this reaction; albeit the efficiency was higher than either *erg26-1<sup>ts</sup> ets1-1* or *ets1-1* cells. Thus, cells carrying the *ets1-1* allele had decreased Erg1p activity. When *ets2-1* cells were fed [ $^{14}$ C]acetate it accumulated in the sterol intermediates, oxido- and dioxidosqualene, whereas in wild-type cells both were efficiently converted to lanosterol (TABLE THREE, WT *versus* *erg26-1<sup>ts</sup> ets2-1* and *ets2-1*). The accumulation of these Erg7p substrates was seen for up to 3 h. Assaying for Erg7p activity in cell extracts using 3S-2,3-[ $^{14}$ C]oxidosqualene as a substrate demonstrated that *erg26-1<sup>ts</sup> ets2-1* (0.15 nmol h<sup>-1</sup> mg<sup>-1</sup>) and *ets2-1* (0.19 nmol h<sup>-1</sup> mg<sup>-1</sup>) cells contained reduced enzymatic activity when compared with wild type (1.0 nmol h<sup>-1</sup> mg<sup>-1</sup>). Interestingly, *erg26-1<sup>ts</sup>* cells (0.32 nmol h<sup>-1</sup> mg<sup>-1</sup>) also contained less Erg7p activity. Thus, cells harboring the *ets2-1* allele had severely weakened Erg7p activity.

Our data suggested that *ets1-1* and *ets2-1* were allelic to *ERG1* and *ERG7*, respectively. If so, replacing the *ERG1* chromosomal locus in *erg26-1<sup>ts</sup> ets1-1* and the *ERG7* chromosomal locus in *erg26-1<sup>ts</sup> ets2-1* with the corresponding wild-type genes should revert the temperature resistance and cause the re-accumulation of 4-carboxysterol intermediates in these cells. We found that this was the case. Thus, we conclude that *ets1-1* and *ets2-1* are recessive alleles of *ERG1* and *ERG7*, respectively. Therefore, we have renamed *ets1-1* as *erg1-1* and *ets2-1* as *erg7-1* for the remainder of the report.

*erg1-1* and *erg7-1* Alleles Harbor Mutations That Affect Activity—To gain more insight into domains required for Erg1p and Erg7p activity, we sequenced *erg1-1* and *erg7-1* alleles. *erg1-1* carried a single G-A nucleotide change at +740 (G247N), resulting in a glycine to aspartic acid change. *erg7-1* harbored two mutations, G-A at +1588 (D530N) and T-A at +1844 (V615E), causing aspartic acid to asparagine and valine to glutamic changes, respectively. We constructed plasmids carrying these alleles (pRS416-*erg1-1* and pRS416-*erg7-1*) and characterized their activities in *erg1-* and *erg7-*null strains. An *erg1* pRS416-*erg1-1* and *erg7* pRS-*erg7-1* cells were viable and accumulated squalene (TABLE TWO, BY4741) and squalene epoxide, respectively, to levels similar to those seen in *erg1-1* and *erg7-1* cells. These mutant alleles were also able to suppress the *ts* phenotype of *erg26-1<sup>ts</sup>* cells, as *erg26-1<sup>ts</sup> erg1* pRS416-*erg1-1* and *erg26-1<sup>ts</sup> erg7* pRS416-*erg7-1* cells were viable at high temperature.

Terbinafine-treated cells show elevated levels of Erg1p (48). The *erg26-1<sup>ts</sup> erg1-1* and *erg1-1* mutants isolated in our study show sensitivity to terbinafine (Fig. 3D and Fig. 4B, W303, 1–2 *versus* 3–4). These mutants also have elevated levels of Erg1p (Fig. 4A; W303, 1–2 *versus* 3–4). *erg1* and *erg26-1<sup>ts</sup> erg1* strains carrying pRS416-*erg1-1* show similar terbinafine sensitivity (Fig. 4B, 5 *versus* 6–7) and elevated levels of Erg1p (Fig. 4A, BY4741, 5 *versus* 6–7). Drug sensitivity differences between strains carrying the original *erg1-1* and those harboring pRS416-*erg1-1* (Fig. 4B, 3–4 *versus* 6–7) were caused by strain background, as BY4741 shows increased resistance to terbinafine in comparison to W303 (Fig. 4C). Thus, null strains harboring recombinant pRS416-*erg1-1* and pRS416-*erg7-1* alleles behave identical to those carrying the original *erg1-1* and *erg7-1* mutations.

*ERG1*, *ERG7*, and *ERG25/26/27* Expression Levels Are Coordinately Regulated in *erg* Mutants—The fact that we observed elevated Erg1p levels in *erg1-1* strains suggests that sterol gene expression may be regulated in response to squalene accumulation (Fig. 4). To test this idea and also determine whether cells respond to the accumulation of squa-

TABLE ONE

## Sterol intermediate accumulation in various ERG26 strains

Sterol intermediate	ERG26	<i>erg26-1</i>	<i>erg26-1ets1-1</i>	<i>erg26-1ets2-1</i>	<i>ets1-1</i>	<i>ets-1</i>
Squalene	0.8	2.0	61.3	0.32	73.7	0.7
Squalene epoxide	ND <sup>a</sup>	ND	ND	44.9	ND	57.6
Zymosterol	19.4	2.8	ND	ND	ND	1.6
Ergosterol	63.1	51.2	38.2	45.1	25.8	35.2
Fecosterol	4.3	1.0	ND	ND	ND	1.1
Episterol	4.1	2.1	ND	1.1	0.2	0.6
Lanosterol	2.9	3.4	ND	5.8	0.1	2.6
Di-methylzymosterol	1.4	1.2	ND	1.4	ND	0.5
4-Carboxy-zymosterol	ND	8.0	ND	ND	ND	ND
4-Methyl-4-carboxy-zymosterol	ND	18.1	ND	ND	ND	ND
Other	3.3	9.5	0.6	4.7	0.2	0.1

<sup>a</sup> Not detected.

TABLE TWO

## Erg1p squalene epoxidase activity in various ERG26 strains

Squalene epoxidase activity was measured using total cell extracts and [<sup>14</sup>C]mevalonic acid. The values represent the average of three independent experiments.

Sterol	W303 <sup>a</sup>				BY4741		
	WT	<i>erg26-1<sup>ts</sup></i>	<i>erg26-1<sup>ts</sup>ets1-1</i>	<i>ets1-1</i>	<i>erg1pRS416-ERG1</i>	<i>erg1pRS416-erg1-1</i>	<i>erg26-1<sup>ts</sup>erg1pRS416-erg1-1</i>
	% total [ <sup>14</sup> C]mevalonic acid incorporated into sterol intermediates						
Squalene	30 ± 2	54 ± 1	94 ± 3	94 ± 2	47 ± 2	93 ± 4	93 ± 5
Squalene epoxide	7.4 ± 0.4	2.0 ± 0.1	0.3 ± 0.1	0.7 ± 0.1	22 ± 4	2.5 ± 0.4	2.5 ± 0.5
Lanosterol	43 ± 1	33 ± 2	1.3 ± 0.1	1.2 ± 0.2	24 ± 3	1.9 ± 0.2	2.7 ± 0.4
Ergosterol	7.8 ± 0.2	2.5 ± 0.2	2.3 ± 0.1	3.0 ± 0.3	ND <sup>b</sup>	ND	ND

<sup>a</sup> Genetic background.<sup>b</sup> None detected.

TABLE THREE

## In vivo Erg7p oxidosqualene cyclase activity in various ERG26 strains

In vivo oxidosqualene cyclase activity was measured as described under "Experimental Procedures." The values are the average of two independent experiments. Duplicate values varied by &lt; 5% of the indicated value.

Strain	Time	Squalene	Oxidosqualene	Dioxidosqualene	Lanosterol	4-Mono methyl sterols	Ergosterol
	<i>h</i>	% total [ <sup>14</sup> C]acetate incorporated into sterol intermediates					
WT	0.5	82.9	3.6	0.8	10.5	1.2	1.0
	3.0	59.9	3.4	1.3	29.5	2.9	2.9
<i>erg26-1<sup>ts</sup></i>	0.5	35.9	7.2	0.6	24.3	5.5	3.4
	3.0	22.2	5.3	2.7	18.8	11.1	39.8
<i>erg26-1<sup>ts</sup>ets2-1</i>	0.5	15.9	58.1	16.4	6.8	1.1	1.6
	3.0	10.9	19.8	44.1	15.8	3.3	6.5
<i>ets2-1</i>	0.5	11.0	42.1	30.2	9.9	1.5	5.6
	3.0	9.5	14.8	46.0	18.7	4.1	6.9

lene epoxides, we examined *ERG1* and *ERG7* expression levels in wild type, *erg1-1*, and *erg7-1*. To determine whether this putative sterol responsive pathway regulates other sterol genes, we also examined the expression levels of *ERG25/ERG26/ERG27*. *ERG25/26/27* together are required for the conversion of 4,4-dimethylzymosterol to zymosterol (Fig. 1) (10). To further define what sterol intermediates may potentially activate the pathway, we looked at expression levels in *erg2-*, *erg6-*, and *erg24-* null cells, as well as wild type cells treated with lovastatin (HMG-CoA reductase inhibitor), fluconazole (Erg11p lanosterol C14-demethylase inhibitor), and amphotericin B or nystatin (ergosterol binding agents) (Fig. 1). *ERG* expression levels were determined using promoter-*LacZ* fusion constructs and/or Northern analysis.

*ERG-LacZ* fusion assays demonstrated that *ERG1* and *ERG25/26/27* expression levels were induced in *erg26-1<sup>ts</sup>*, *erg1-1*, and *erg7-1* cells, whereas *ERG7-LacZ* induction was observed only in *erg7-1* cells (Fig. 5, A and B, wild type, hatched bars; *erg26-1<sup>ts</sup>*, black bars; *erg1-1*, gray bars;

*erg7-1*, white bars). Northern analysis confirmed these results, while revealing that *ERG7* expression was actually regulated similarly to other *ERG* genes tested (Fig. 5C). Thus *ERG1*, *ERG7*, and *ERG25/26/27* expression levels are coordinately regulated in response to squalene, squalene epoxide, and 4-carboxysterol accumulation. More importantly, we found that *ERG* expression regulation extends to and is activated by blocks in Erg2p (*erg2*), Erg6p (*erg6*), Erg24p (*erg24*), Hmg1p/Hmg2p (lovastatin), and Erg11p (fluconazole) functions (Fig. 5, D and E). *ERG1*, *ERG7*, and *ERG25/26/27* expression levels were induced to varying degrees in null strains or in wild-type cells treated with lovastatin or fluconazole. Induction was not observed in cells treated with amphotericin B or nystatin (Fig. 5E).

*Sterol Responsive ERG Expression Induction Requires Upc2p and Ecm22p Transcription Factors*—We wanted to begin to define the cell factors functioning to regulate *ERG* expression in response to blocks in sterol biosynthesis. We initiated these studies by examining lovastatin-

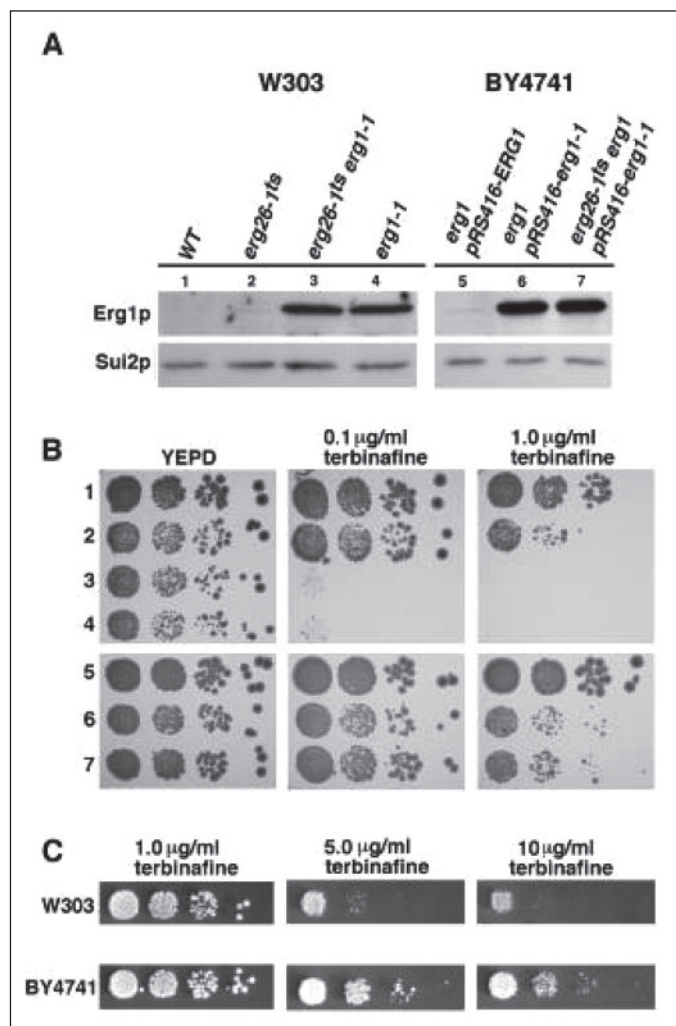


FIGURE 4. Strains harboring recombinant *erg1-1* have phenotypes indicative of weakened Erg1p activity. A, total cell extract protein was obtained from the indicated strains and was resolved by SDS-PAGE and subjected to Western analysis using anti-Erg1p polyclonal antibodies. *Sul1p*, loading control. B, 10-fold serial dilutions of the indicated strains were grown on YEPD plates in the absence and presence of the indicated concentrations of terbinafine. Strain row numbers in C correspond to strain column numbers in B. C, 10-fold serial dilutions of W303 and BY4741 were grown on YEPD plates in the presence of the indicated concentrations of terbinafine.

induced expression induction in cells lacking either one or both transcription factors, Upc2p and Ecm22p, lacking the transcriptional repressor, Mot3p (49–51), or lacking the nuclear protein, Yer064cp, previously shown to be required for *ERG* induction (52). Cells lacking both Upc2p and Ecm22p, or those lacking Yer064cp, were no longer able to induce the expression of *ERG1/7/25/26/27* in response to lovastatin treatment (Fig. 6). The loss of Mot3p, Upc2p, or Ecm22p functions alone had no effect. Identical results were obtained when we examined fluconazole-induced expression induction. Thus, the loss of both Upc2p and Ecm22p functions, and not either alone, was required to eliminate sterol responsive expression regulation.

## DISCUSSION

Our results demonstrate that the expression of yeast *ERG1*, *ERG7*, and *ERG25/26/27* genes are coordinately regulated in response to multiple blocks in sterol biosynthesis. Strikingly, Northern analysis revealed that the degree of induction was not the same for all mutant strains tested. These results suggest the possibility that the pathway controlling expression of these and other *ERG* genes may have the ability to be

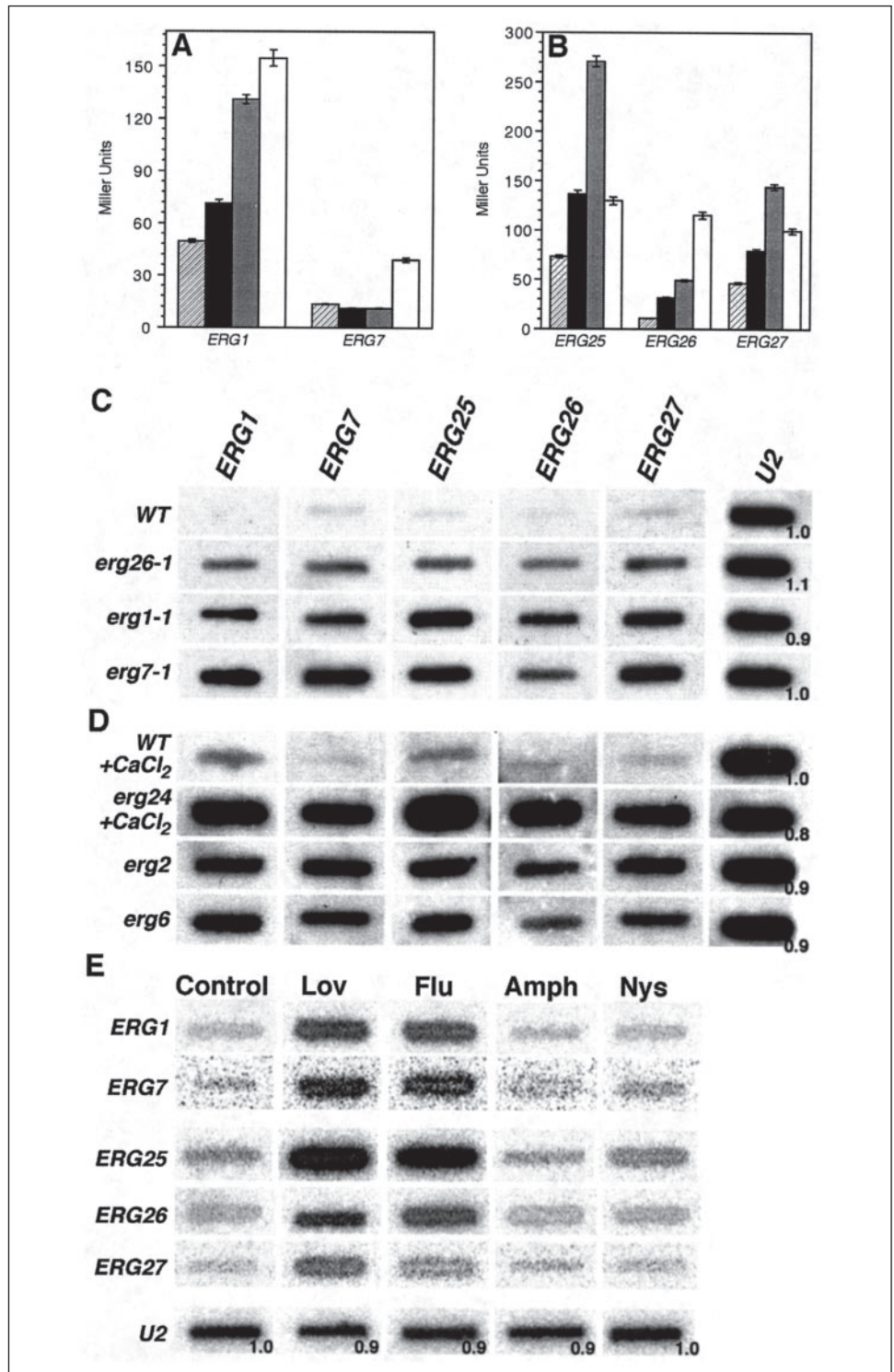
activated to different levels, depending on what specific sterol intermediate accumulates, or to what extent ergosterol levels are depleted. By using the HMG-CoA reductase inhibitor, lovastatin, we showed that the transcription factors, Upc2p and Ecm22p are required for expression regulation.

The genes and/or cDNAs for human *ERG1* and *ERG7* have been cloned and the Erg7 oxidosqualene cyclase protein has been crystallized (53–57). The *hERG1* promoter contains a consensus SRE that acts as an activator sequence in cells overexpressing SREBP, and a nuclear factor Y binding site that is required for sterol-dependent regulation (58). Whether these sequences act together in response to sterol levels or independently is not known. M'Baya *et al.* (59) has shown that yeast *ERG1* is regulated in response to changes in sterol levels. *ERG1* contains a Vik and Rine (24) 7-bp SRE, and a sterol responsive promoter sequence, comprised of two 6-bp direct repeats, that is required for terbinafine- and ketoconazole-dependent induction of *ERG1* expression (60). Presently, little is known about the transcriptional regulation of *ERG7* in yeast. Interestingly, the *ERG7* promoter does not contain a "putative" consensus yeast SRE element.

The cDNAs or genes for human and mouse *ERG25/26/27* have been cloned (61–65). There is a report that *ERG25* is regulated through an SREBP-dependent mechanism in the vascular wall of pigs, but little else is known about expression regulation of mammalian *ERG25/26/27* (66). We showed that a sterol responsive pathway coordinately regulates the expression of these genes. *ERG25* does contain three yeast SRE sites (24), while *ERG26* and *ERG27* lack this sequence. The transcription factors, Ecm22p and Upc2p were shown to bind to the SREs of *ERG2* and *ERG3*, and they most likely regulate the expression of these genes in response to lovastatin treatment and inhibition of HMG-CoA reductase activity (24).

These results suggest that *ERG25* expression should be regulated in the same manner, through the binding of Upc2p/Ecm22p to SREs in the *ERG25* promoter. So then how can we reconcile the fact that *ERG26* and *ERG27* lack SREs, yet they are coordinately regulated along with *ERG25* in response to changes in sterol levels? One possibility is that *ERG26* and *ERG27* contain a novel Ecm22p and/or Upc2 binding site(s) that has yet to be elucidated. Another explanation is that two independent pathways exist that both converge on *ERG* genes to regulate their expression in response to changes in sterol levels. Recall that *hERG1* contains both SRE and nuclear factor Y binding sites that may act independently to regulate expression in response to changes in sterol levels. We are currently determining the promoter sequences within *ERG26* and *ERG27* required for sterol responsive expression regulation.

The Erg1p squalene epoxidase converts squalene to 2,3-oxidosqualene (2, 11). The reaction requires oxygen and FAD. The Erg7p oxidosqualene cyclase then converts the Erg1p product, 2,3-oxidosqualene, to lanosterol (2, 11). We mapped a single mutation in the yeast *ERG1* gene (G247D) that causes increased *ERG1* expression, elevated levels of Erg1p, altered SBI sensitivities and weakened enzymatic activity. Some of these phenotypes have been seen in other *erg1* mutants or in cells treated with terbinafine (47, 48). The G247D mutation resides within a conserved domain of Erg1p that is necessary for monooxygenase activity (67, 68). Interestingly, a terbinafine resistant mutant of Erg1p (L251F) in *S. cerevisiae* has been isolated, which carries a mutation within this same region (67). Thus, terbinafine resistance and sensitivity can map within the same conserved region. We mapped two mutations in Erg7p (D530N, V615E) that together were necessary for suppressing the *ts* and 4-carboxysterol accumulation of *erg26-1<sup>ts</sup>* cells. *erg7* alleles harboring either mutation alone were unable to suppress these phenotypes. The



**FIGURE 5. Multiple ERG genes are coordinately regulated by a sterol responsive pathway.** A, steady state levels of *ERG1-LacZ* and *ERG7-LacZ* promoter activities were assayed in the indicated strains using the substrate, CPRG. B, steady state levels of *ERG25-LacZ*, *ERG26-LacZ*, and *ERG27-LacZ* promoter activities were assayed in the indicated strains using the substrate, CPRG. C and D, mRNA expression levels were determined for the indicated ERG genes by Northern slot blot hybridization. E, mRNA expression levels were determined in wild-type cells grown in the absence and presence of 50  $\mu\text{g/ml}$  lovastatin for 12 h, or 16  $\mu\text{g/ml}$  fluconazole, 0.25  $\mu\text{g/ml}$  amphotericin B, or 10  $\mu\text{g/ml}$  nystatin for 3 h. Wild type, hatched bars; *erg26-1<sup>ts</sup>*, black bars; *erg1-1*, gray bars; *erg7-1*, white bars; U2, loading control. The numbers represent relative density values for U2, where all values are compared with the wild-type control. *erg24*-null cells require 200 mM CaCl<sub>2</sub> for viability.

mutations reside within a large conserved domain required for sterol cyclization (57).

Our *in vitro* enzymatic assays demonstrated that *erg26-1<sup>ts</sup>* cells had weakened Erg1p and Erg7p activities (TABLES TWO and THREE). In the case of Erg1p, we found that mutant cell extracts accumulated ~2-fold higher levels of the Erg1p substrate, squalene, and produced less lanosterol than wild-type cells. Mammalian mevalonate kinase is inhibited by specific cholesterol biosynthetic and nonsterol isoprene intermediates (69). Multiple studies have demonstrated that yeast and

mammalian phospholipid biosynthetic enzyme activities are regulated by numerous lipid species (70–75). Thus, the 4-carboxysterols accumulating in *erg26-1<sup>ts</sup>* cells could be directly interacting with and competitively or non-competitively inhibiting Erg1p activity. Our results bring up the possibility that regulation of *ERG26* mRNA levels and/or Erg26p enzymatic activity may represent a temporally efficient way for yeast cells to regulate other sterol biosynthetic enzymes through changes in the levels of 4-carboxysterols in cells. Lipid-dependent regulation of Ergp activities adds a second level of sophistication to mechanisms reg-



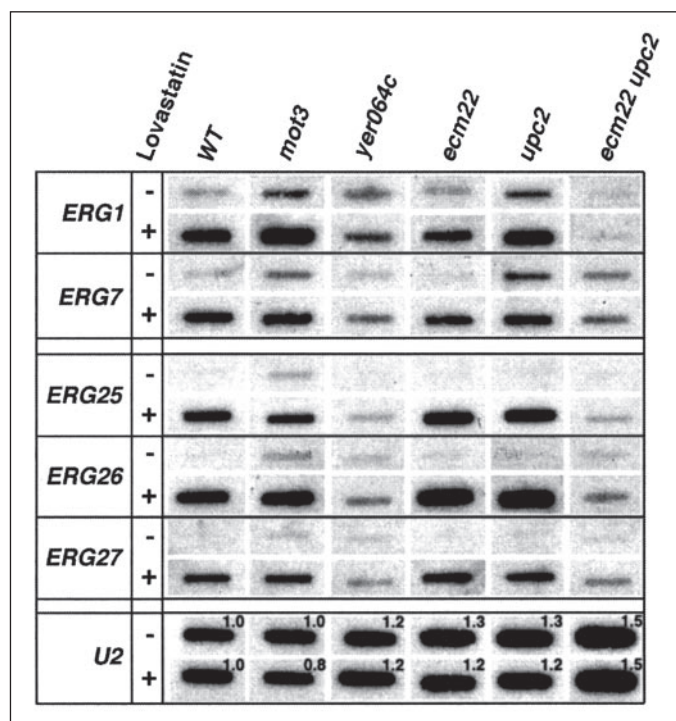


FIGURE 6. Upc2p and Ecm22p function to regulate ERG1, ERG7, and ERG25/26/27. mRNA expression levels were determined for the indicated ERG genes by Northern slot blot hybridization. Lovastatin concentration was 50  $\mu$ g/ml. U2, loading control. The numbers represent relative density values for U2, where all values are compared with the wild-type control in the absence of lovastatin.

ulating the sterol pathway. It may also help to explain why ERG25/26/27 are coordinately regulated with other sterol biosynthetic genes.

Gachotte *et al.* (28) showed that *erg27* mutants do not accumulate the predicted 3-ketosterols, but rather those intermediates that are seen in cells harboring defects in Erg7p activity. In fact, *erg27* mutants have decreased levels of Erg7p activity, which can be partially suppressed by ectopic expression of an Erg27p-HA chimera (76). Moreover, Erg7p associates with Erg27p in lipid particles, and this association seems to be necessary for organelle localization and for protein stability. Thus, Erg27p and Erg7p may form a complex *in vivo*, and that decreased Erg7p activity in *erg27* mutants arises from mislocalization and proteolytic degradation of the protein because of the loss of this complex (76). Studies have demonstrated physical interactions between Erg25p/Erg26p/Erg27p (25, 77). The *erg26-1<sup>ts</sup>* allele could interfere in some way with the interaction between Erg27p and Erg7p, causing decreased activity. However, *erg26* cells do not accumulate those sterols seen in *erg27* cells (25, 26). An alternative explanation is that the 4-carboxysterols in *erg26-1<sup>ts</sup>* cells directly interact with Erg7p and inhibit its activity. If this type of lipid-dependent regulation is subtle, it may only be unmasked using *in vitro* enzymatic assays. Enzymatic assays using purified Erg1p and Erg7p preparations will help to resolve these issues.

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78. <sup>2</sup> To whom correspondence should be addressed: Dept. of Biochemistry and Molecular Biology, Drexel University College of Medicine, Philadelphia, PA 19102. Tel.: 215-762-1941; Fax: 215-762-4452; E-mail: JN27@drexel.edu.

## **Characterizing Sterol Defect Suppressors Uncovers a Novel Transcriptional Signaling Pathway Regulating Zymosterol Biosynthesis**

Melody Germann, Christina Gallo, Timothy Donahue, Reza Shirzadi, Joseph Stuke, Silvia Lang, Christoph Ruckstuhl, Simonetta Oliaro-Bosso, Virginia McDonough, Friederike Turnowsky, Gianni Balliano and Joseph T. Nickels, Jr.

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