Erg28p is a key protein in the yeast sterol biosynthetic enzyme complex

Caiqing Mo and Martin Bard[1]

Biology Department, Indiana University-Purdue University Indianapolis, Indianapolis, IN 46202

Abstract **Previously, a microarray expression study in the yeast *Saccharomyces cerevisiae* indicated that the *ERG28* gene was strongly coregulated with ergosterol biosynthesis. Subsequently, Erg28p was shown to function as an endoplasmic reticulum transmembrane protein, acting as a scaffold to tether the C-4 demethylation enzymatic complex and also to interact with a downstream enzyme, Erg6p. To understand all possible protein interactions involving Erg28p in sterol biosynthesis, a yeast two-hybrid system designed to assess interactions between membrane proteins was used. The Erg28p fusion protein was used as bait to assess interactions with all 14 sterol biosynthetic proteins in a pairwise study based on two reporter systems as well as Western blots demonstrating the release of a transcription factor. Our results indicated that Erg28p not only interacted with the C-4 demethylation enzymes and Erg6p but also with Erg11p and Erg1p. Interactions between Erg28p and seven ergosterol biosynthetic enzymes were confirmed by coimmunoprecipitation experiments. Furthermore, by comparing the reporter gene expression levels, we demonstrate that Erg28p is most closely associated with Erg27p, Erg25p, Erg11p, and Erg6p and less with Erg26p and Erg1p. Based on these results, we suggest that many if not all sterol biosynthetic proteins may be tethered as a large complex.**—Mo, C., and M. Bard. **Erg28p is a key protein in the yeast sterol biosynthetic enzyme complex. *J. Lipid Res.* 2005.** 46: **1991–1998.**

Supplementary key words ergosterol biosynthesis • membrane proteins • split-ubiquitin membrane yeast two-hybrid system

Sterols are essential components of eukaryotic cell membranes. The yeast *Saccharomyces cerevisiae* accumulates ergosterol, the sterol equivalent of cholesterol in animals (1) and sitosterol in plants (2), as the end product sterol. In yeast, 14 genes and 15 enzymatic steps are involved in ergosterol biosynthesis (**Fig. 1**) (3). An important aspect of sterol synthesis, as provided by yeast as a model system, is that many of the reactions are identical to those of the cholesterol pathway in human cells (4). This is true of the prelanosterol steps and many of the distal steps leading to ergosterol formation. Thus, *S. cerevisiae* has been used extensively in the study of the sterol biosynthetic pathway as well as in studies involving the physiological features of sterol molecules required for various cellular processes (5–7).

In a yeast microarray expression profile study, the gene *YER044C* encoding a 148 amino acid protein with two transmembrane domains, now designated *ERG28*, was found to be highly coregulated with other ergosterol biosynthetic genes (8). An *ERG28* deletion was found to be slow growing. Loss of Erg28p does not lead to the loss of end product sterol but rather reduces ergosterol levels to approximately one-third of wild-type levels (9). *ERG28* is conserved in *S. cerevisiae*, *Schizosaccharomyces pombe*, *Arabidopsis thaliana*, mouse, and human (10), and in humans, for unexplained reasons, it appears to be highly expressed in adult testis tissue (11).

In an *ERG28* deletion strain, gas chromatographic analyses indicated significant accumulation of sterol precursors that were identified as carboxylic acid sterol and 3-keto sterol intermediates, normally accumulating in *ERG26* and *ERG27* mutant strains; thus, Erg28p affects the normal process of C-4 demethylation (9). We subsequently demonstrated that Erg28p is an endoplasmic reticulum (ER) transmembrane protein, working as a transmembrane scaffold to tether Erg27p and possibly other C-4 demethylation proteins (Erg25p, Erg26p) to form a demethylation complex in the ER (12). More recent studies also indicated that Erg28p not only anchors the C-4 demethylation enzyme complex to the ER but also acts as a tether to Erg6p, a side chain transmethylation enzyme required for a downstream reaction in sterol biosynthesis (13) (Fig. 1).

Because of the prevalence and conservation of *ERG28* among fungi, animal, and plant sterol biosynthetic path-

Abbreviations: 3-AT, 3-aminotriazole; CSM, complete synthetic medium; Cub, C-terminal ubiquitin; ER, endoplasmic reticulum; HA, peptide from human hemagglutinin; Nub, N-terminal ubiquitin; TF, transcription factor containing protein A-LexA-VP16; X-Gal, 5-bromo-4-chloro-3-indolyl-β-D-galactoside; YTH, yeast two-hybrid.

[1] To whom correspondence should be addressed.
e-mail: mbard@iupui.edu

Manuscript received 19 April 2005 and in revised form 3 June 2005 and in re-revised form 23 June 2005.

Published, JLR Papers in Press, July 1, 2005.
DOI 10.1194/jlr.M500153-JLR200

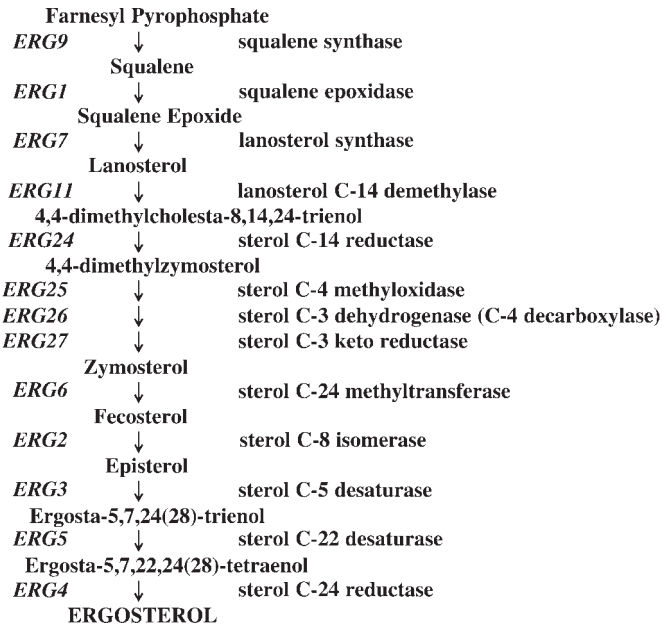

Fig. 1. The ergosterol biosynthetic pathway starting with *ERG9*, the first gene in the dedicated sterol pathway.

ways (10), we suggest that *ERG28*'s unique function in sterol biosynthesis may be to tether the entire biosynthetic complex of enzymes into a single functioning unit. No enzyme in any sterol biosynthetic pathway has been demonstrated to act as a sterol binding protein, and no common sterol binding motif shared by the biosynthetic enzymes has been found. To systematically delineate the role of Erg28p, a strategy designed to evaluate all Erg28p interaction partners was adopted.

Typical biochemical methods, such as coimmunoprecipitation, copurification, or cross-linking, have been used to investigate protein complexes. However, because of the limitations of each of these biochemical methods, it is difficult to obtain reliable information regarding partners in membrane-protein interaction assays (14). For example, Gavin et al. (15) used tandem affinity copurification and mass spectrometry in a large-scale approach to characterize multiprotein complexes in *S. cerevisiae*, and among 1,799 encoded proteins they chose to study, only 40 proteins that were integrally or peripherally associated with membranes were purified, and none was involved in ergosterol biosynthesis. Another study by Ho et al. (16) using immunoaffinity purification based on the flag epitope tag gave similar results.

To study the protein-protein interactions, alternative genetic methods have been developed, and the most powerful genetic method for the study of protein-protein interactions is the yeast two-hybrid (YTH) system (17), which is based on the reconstitution of a functional transcription factor (TF) through a defined protein-protein interaction. Although various modifications of the YTH system have been described (18–20), the analysis of interactions between membrane proteins has remained a significant challenge because of the hydrophobic nature of membrane proteins as well as the fact that integral and membrane-associated proteins often undergo posttranslational modifications or oligomerizations via interactions between transmembrane domains (14). A strategy called the split-ubiquitin membrane YTH system was recently described (14) that takes advantage of the split-ubiquitin approach first described by Johnsson and Varshavsky (21). The N- and C-terminal halves of ubiquitin (22) [split-ubiquitin: N-terminal ubiquitin (Nub) and C-terminal ubiquitin (Cub)] were fused to bait and prey proteins, respectively. As two membrane proteins physically associate in vivo, Nub and Cub reconstitute, leading to proteolytic cleavage at a GGXX site by ubiquitin-specific binding proteases, releasing a TF fused to the Cub polypeptide. The released TF (protein A-LexA-VP16) can then activate two reporter genes (*LacZ* and *HIS3*). This system had been used extensively to demonstrate novel protein-protein interactions (23–26).

In this study, the Erg28p-Cub-TF fusion protein, consisting of Erg28p, the Cub, and a TF, was fused as the bait protein to assess interactions with 14 prey yeast Erg proteins [fused to two copies of the human hemagglutinin (HA) peptide followed by the N-terminal ubiquitin polypeptide (ErgXp-HA-Nub)]. Interactions between Erg28p and all 14 yeast Erg proteins were studied pairwise, and new interaction partners were confirmed by coimmunoprecipitation.

MATERIALS AND METHODS

Yeast strains and growth conditions

The L40 (*MATa trp1 leu2 his3 LYS2::lexA-HIS3 URA3::lexA-lacZ*) yeast strain containing the *HIS3* and *LacZ* reporters was cotransformed with the pERG28-Cub-TF (bait plasmid) and all 14 ergosterol biosynthetic gene constructs (prey plasmids containing pERGX-HA-Nub, where X represents individual ergosterol genes). L40 transformants containing pERG28-Cub-TF were grown on complete synthetic medium (CSM) containing 0.67% yeast nitrogen base, 2% glucose, and 0.8 g/l CSM-Leu (amino acid and nitrogen base supplements; Bio101, Vista, CA). L40 transformants containing pERG28-Cub-TF and pERGX-HA-Nub were grown on CSM-Leu-Trp synthetic dropout medium to ensure retention of the bait and prey plasmids, respectively. CSM-Leu-Trp-His with varying concentrations of 3-aminotriazole (3-AT) was used to assess the strength of bait and prey interactions in spot tests. R712 (*MATa erg28*::*kanMX4 his3Δ1 leu2Δ0 met15Δ0 ura3Δ0*), a previously described *erg28* deletion strain (9), was transformed with pERG28-Myc plasmid (12) and used for immunoprecipitation studies.

Primer design and PCR cloning

The bait vector, pCYC-BAIT-Cub-TF (pCub), and prey vector, pADH-PREY-HA-Nub (pNub), were kindly provided by Dr. Igor Stagljar (14). pCub is a CEN plasmid containing *LEU2* and a weak yeast *CYC1* promoter, multiple cloning sites followed by the C-terminal Cub sequence, and a TF consisting of protein A, LexA, and VP16. The Nub plasmid is a 2μ vector containing the *TRP1* marker, a strong yeast *ADH1* promoter, multiple cloning sites followed by two HA tags, and the N-terminal Nub sequence.

Primers for PCR cloning of *ERG28* into bait vector and prey vector, and primers for PCR cloning of all of the ergosterol biosynthetic genes into prey vectors, are listed in **Table 1**. For the reverse primer, stop codons for each ergosterol biosynthetic gene were deleted and a single base pair added to maintain the cor-

TABLE 1. Primers used to design bait and prey constructs

Genes	Primer Sequences (5′ to 3′)	Digestion Sites	Fusion Protein
			kD
ERG28	F: GGCTGCAG**CATATG**TTCAGCCTACAAGACG	*Pst*I-*Hind*III[a]	70.0
	R: GGAAGCTT**CGGATCC**CCAAGCAACACCAGTGTA	*Nde*I-*Bam*HI	26.0
ERG9	F: GGCTGCAG**CATATG**GGAAAGCTATTACAATTG		
	R: GGAAGCTTC**GGATCC**CCGCTCTGTGTAAAGTGTATA	*Nde*I-*Bam*HI	60.5
ERG1	F: GGCTGCAG**CCATGG**ATGTCTGCTGTTAACGTTG		
	R: GGAAGCTTC**GGATCC**CACCAATCAACTCACCAAAC	*Nco*I-*Bam*HI	64.0
ERG7	F: CTTTTTCTGCACAATATTTCAAGCTATACCAAGCATACAA-TCAACT**CATATG**GCCATGGATGACAGAATTTTATTCTGAC	Cotransformation of prey vector by gap repair[b]	92.4
	R: GGAACATCGTATGGGTACTGGATCTGAGCGTAATCTGGAAC-ATCGTATGGGTACT**GGATCC**CAAGCGTATGTGTTTCATATG	*Nde*I-*Bam*HI	
ERG11	F: GGCTGCAG**CATATG**TCTGCTACCAAGTCAAT		
	R: GGAAGCTTC**GGATCC**CGATCTTTTGTTCTGGATTTCT	*Nde*I-*Bam*HI	69.5
ERG24	F: GGTCTAGA**CCATGG**ATGGTATCAGCTTTGAATCC		
	R: GGCTGCAGC**GGATCC**CATAAACATATGGAATGATCTTG	*Nco*I-*Bam*HI	59.4
ERG25	F: GGCTGCAG**CATATG**TCTGCCGTTTTCAACAA		
	R: GGAAGCTTC**GGATCC**CGTTAGTCTTCTTTTGAGCATT	*Nde*I-*Bam*HI	45.3
ERG26	F: GGCTGCAG**CATATG**TCAAAGATAGATTCAGTT		
	R: GGAAGCTTC**GGATCC**CCAAACCTTCGTCCATCCA	*Nde*I-*Bam*HI	47.5
ERG27	F: GGCTGCAG**CATATG**ATGAACAGGAAAGTAGCTAT		
	R: GGAAGCTTC**GGATCC**CAATGGGGGTTCTAGTTTCA	*Nde*I-*Bam*HI	46.5
ERG6	F: GGCTGCAG**CATATG**ATGAGTGAAACAGAATTGAG		
	R: GGAAGCTTC**GGATCC**CTTGAGTTGCTTCTTGGGAA	*Nde*I-*Bam*HI	52.3
ERG2	F: GGCTGCAG**CCATGG**ATGAAGTTTTTCCCACTCC		
	R: GGAAGCTTC**GGATCC**CGAACTTTTTGTTTTGCAACAA	*Nco*I-*Bam*HI	33.7
ERG3	F: GGCTGCAG**CATATG**GATTTGGTCTTAGAAGT		
	R: GGAAGCTTC**GGATCC**CGTTGTTCTTCTTGGTATTTG	*Nde*I-*Bam*HI	51.5
ERG5	F: GGACTAGT**CCATGG**ATGAGTTCTGTCGCAGAAA		
	R: GGACTAGTC**GGATCC**CTTCGAAGACTTCTCCAGTA	*Nco*I-*Bam*HI	70.0
ERG4	F: GGTCTAGA**CATATG**GCAAAGGATAATAGTGA		
	R: GGAAGCTTC**GGATCC**CGAAAACATAAGGAATAAAGAC	*Nde*I-*Bam*HI	64.8

F, forward; R, reverse. Digestion sites for insertion into bait and prey vectors are underlined, and added base pairs to maintain correct reading frames are indicated in boldface.

[a] Primers for bait construct; all other primer sequences are for prey constructs.

[b] From ref. 27, 28.

rect frame with the Nub or Cub sequences. Primer pairs and restriction sites for insertion of encoded open reading frames into bait and prey vectors are also listed in Table 1. Because no suitable restriction sites existed for inserting *ERG7* into the pNub vector, the PCR product of the *ERG7* gene was cloned into the prey vector by gap repair, in which the *ERG7* gene containing *Nco*I and *Bam*HI restriction sites as well as an additional 50 bp of DNA homologous to the ends of the gapped vector were cotransformed into L40 yeast cells (27, 28). Plasmid was then rescued from transformed yeast cells.

Complementation test for the pERG28-Cub-TF construct

To determine the functionality of the pERG28-Cub-TF construct, pERG28-Cub-TF was transformed into the *erg28* deletion strain, R712 (9). Complementation of the *erg28* growth defect by pERG28-Cub-TF was assessed by spot plates (8) and gas chromatographic analyses (9).

Cub and Nub fusion protein screen

Yeast L40 cells transformed with the bait construct (pERG28-Cub-TF) or with various prey constructs (pERGX-HA-Nub) were grown in CSM-Leu or CSM-Trp liquid medium, respectively (1.5 ml), pelleted after 24 h of growth, and resuspended in 50 μl of 1× SDS-PAGE loading buffer (0.0625 M Tris-HCl, pH 6.8, 2% SDS, 10% glycerol, 1% β-mercaptoethanol, 0.001% bromophenol blue, 1× SSB) (12). Cell suspensions were vortexed, boiled for 5 min, cooled to room temperature, and pelleted; aliquots of supernatant (10–40 μg of total proteins) were loaded onto SDS-PAGE gels and subsequently transferred to nitrocellulose membranes according to standard procedures (29). Nitrocellulose membranes were blocked using 5% nonfat milk/PBS-T buffer for 1 h and then incubated with anti-HA HRP (500× dilution; Santa Cruz Biotechnology, Santa Cruz, CA) to detect the ErgXp-HA-Nub fusion protein or with anti-VP16 HRP (300× dilution; Santa Cruz Biotechnology) for Erg28p-Cub-TF fusion protein detection as well as detection of the released TF (a truncated version of the Erg28p-Cub-TF fusion protein; VP16 is a truncated component of TF: protein A-LexA-VP16).

Growth differentiation and spot tests

To systematically determine which ergosterol biosynthetic proteins interact with Erg28p, the pERG28-Cub-TF plasmid was transformed into L40 along with 14 pERGX-HA-Nub fusion constructs as well as the empty prey pNub vector used as a negative control. All doubly transformed colonies were plated onto CSM-Leu-Trp plates. For each of the 15 transformations, 6 colonies were spot-plated onto petri plates (150 mm × 15 mm) containing CSM-Leu-Trp and CSM-Leu-Trp-His plus varying concentrations of 3-AT (0–5 mM). Each colony was spotted using 2 μl from a 2×10^7 cells/ml cell suspension. Plates were incubated at 30°C for 48 h and scored.

β-Galactosidase assays

Approximately 1 μl of a cell suspension (2×10^7 cells/ml) was placed onto sterilized nitrocellulose membranes that were then overlaid onto CSM-Leu-Trp plates. To assess interactions between pERG28-Cub-TF and 14 pERGX-HA-Nub constructs, six colonies were spotted. After 48 h of growth at 30°C, β-galactosidase activ-

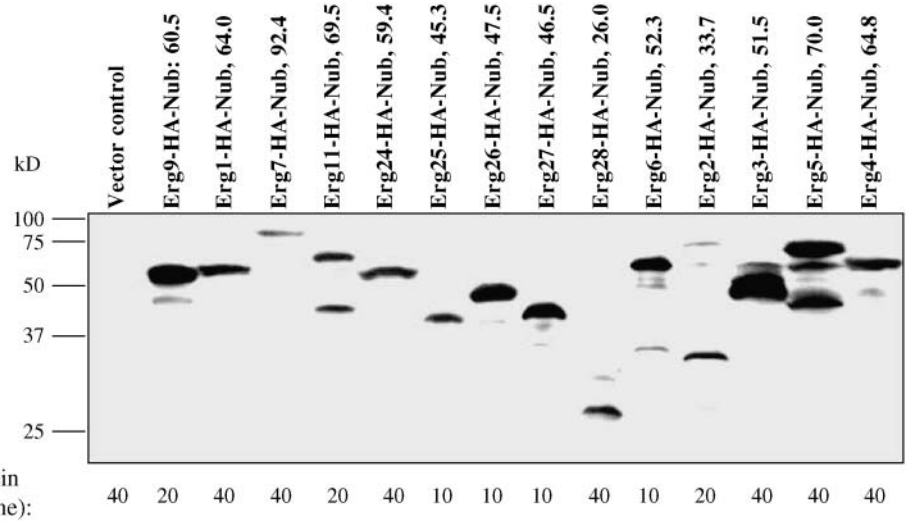

Fig. 2. Anti-human hemagglutinin (HA) Western blot of each ErgXp-HA N-terminal ubiquitin (Nub) fusion protein (where X represents an ergosterol biosynthetic protein). The lane order is arranged according to the sequence of reactions in the ergosterol biosynthetic pathway. The size of the fusion protein is indicated for each prey construct. The amount of protein extract loaded for each construct is indicated in micrograms.

ity for each colony was assayed as follows: the nitrocellulose membrane was peeled from the solid medium plates and dipped into liquid nitrogen for 2 min, then carefully placed onto a 3MM round filter paper in an empty dish. β-Galactosidase Z buffer (2.5 ml) was added onto the 3MM filter paper, and membranes were then soaked with 5-bromo-4-chloro-3-indolyl-β-D-galactoside (X-Gal) (139 μl of X-Gal made as a 20 mg/ml stock in DMSO). The reaction was allowed to proceed for 10–30 min at 30°C in the dark. Z-buffer (30) containing 0.1 M Na-phosphate, pH 7.2, 0.75 g/l KCl, and 0.246 g/l $MgSO_4$ was filter-sterilized, and 6.6 μl of β-mercaptoethanol was added.

Membrane protein preparation, solubilization, and immunoprecipitation

The preparation, solubilization, and immunoprecipitation of yeast membrane proteins were performed as described previously (12). Essentially, membrane proteins were solubilized at 1 mg/ml with 2 mM sucrose monolaurate. Previously described anti-Erg1p (31), anti-Erg6p (32), anti-Erg7p (33), anti-Erg11p (13), and anti-Erg25p (34) were used to coimmunoprecipitate Erg28p-Myc. Anti-Lcb1p (35) was used as a loading control. Antibodies to Erg2p and Erg3p were prepared using the last 20 amino acids of Erg2p (RTVYLTARDMGKNLLQNKKF) and 20 amino acids in the middle of Erg3p (DPKLRDAKETWDAQVKEVEH), respectively. These two peptides were synthesized and used to immunize rabbits by Proteintech Group (Chicago, IL). Anti-Erg2p and anti-Erg3p sera were collected, and the expected 24 kDa Erg2p band and a 43 kDa Erg3p band were observed (data not shown). Solubilized membrane protein (1 ml at 1 mg/ml) was diluted with microsomal buffer and incubated with anti-Lcb1p (200× dilution), anti-Erg1p (200× dilution), anti-Erg2p (150× dilution), anti-Erg3p (100× dilution), anti-Erg6p (100× dilution), anti-Erg7p (100× dilution), anti-Erg11p (150× dilution), and anti-Erg25p (150× dilution) for 2 h at 4°C followed by the addition of 40 μl of protein A/G plus agarose (Santa Cruz Biotechnology). Samples were further incubated for 2 h at 4°C. Precipitates were washed three times with 500 μl of 50 mM HEPES (pH 7.5) and resuspended in 40 μl of 2× SDS-PAGE sample loading buffer, and 15 μl of each sample was subjected to SDS-PAGE and immunoblot analysis. Anti-Myc-peroxidase (clone 9E10; Roche Diagnostics, Indianapolis, IN) was used to identify which proteins coimmunoprecipitated with Erg28p-Myc.

RESULTS

Cloning of the prey pERGX-HA-Nub constructs

PCR products of the *ERG28* bait and the 14 preys (14 ergosterol biosynthetic enzyme open reading frames) were digested and cloned into the bait (pCub) and prey (pNub) vectors, respectively (14). The bait construct (pERG28-Cub-TF) and all of the prey constructs (pERGX-HA-Nub) were transformed into the L40 yeast reporter strain and grown on CSM-Leu and CSM-Trp, respectively. For the Erg28p-Cub-TF fusion protein, a 70 kDa band was detected by anti-VP16 antibody in L40 (pERG28-Cub-TF) cells (see Fig. 5 below). For each ErgXp-HA-Nub fusion protein, fusion proteins of the predicted size were detected by Western blotting using anti-HA antibody (**Fig. 2**).

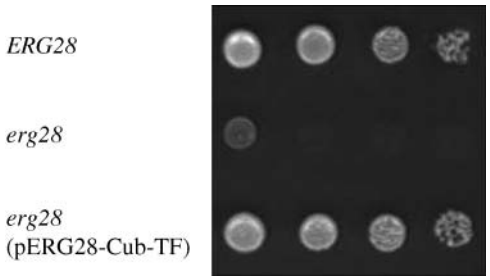

Fig. 3. The pERG28-C-terminal ubiquitin-transcription factor (Cub-TF) plasmid complements the *erg28* deletion growth defect in yeast complete synthetic medium (CSM). The *ERG28* and *erg28* strains were grown on CSM, whereas the pERG28-Cub-TF plasmid-containing *erg28* strain was grown on CSM-Leu medium.

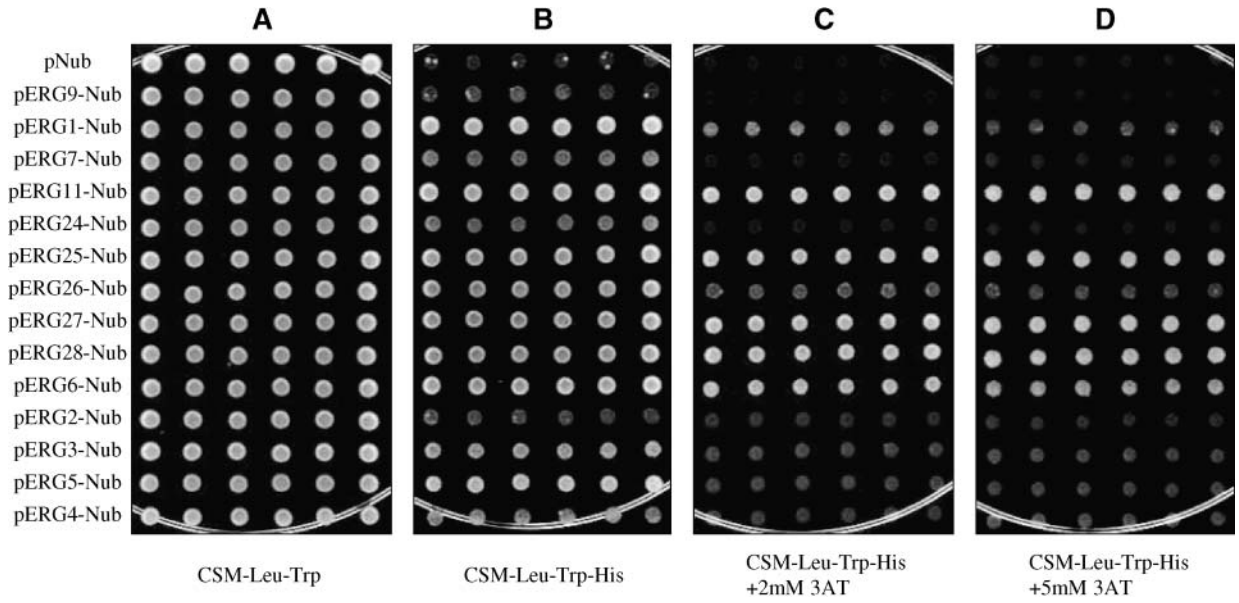

Fig. 4. Spot test assay using the *HIS3* reporter to assess interactions between Erg28p-Cub-TF and various ErgXp-HA-Nub preys. Media used to assess the strength of interaction between bait and prey are as follows: CSM-Leu-Trp, growth control (A); CSM-Leu-Trp-His (B); CSM-Leu-Trp-His + 2 mM 3-aminotriazole (3-AT) (C); and CSM-Leu-Trp-His + 5 mM 3-AT (D). Six colony replicates containing both bait and prey constructs were spotted onto each of the four media.

Complementation test for the Cub construct

The pERG28-Cub-TF plasmid construct was transformed into an *erg28* deletion strain, R712, to determine whether the Erg28p-Cub-TF fusion protein might complement an *ERG28* null allele. **Figure 3** demonstrates that the pERG28-Cub-TF plasmid restores the wild-type growth rate to an *erg28* mutant strain. Gas chromatographic analyses of sterols from the transformed strain also indicated a wild-type sterol profile (data not shown).

Erg28p interacts with enzymes in ergosterol biosynthesis using spot plate assays

Figure 4 demonstrates protein-protein interactions between the bait construct Erg28p and all 14 biosynthetic enzymes in the dedicated ergosterol pathway. Figure 4A demonstrates growth on plates with no selection except for plasmid retention (CSM-Leu-Trp plates). Figure 4B demonstrates growth on CSM-Leu-Trp-His plates in which the *HIS3* reporter activity is dependent upon an interaction between the bait and prey proteins. Figure 4C, D contain increasing concentrations of 3-AT (2 and 5 mM, respectively, of the *HIS3* inhibitor), delineating an increased requirement for *HIS3* reporter activity and therefore an increased affinity of interaction between bait and prey. These results indicate that Erg28p strongly interacts with Erg11p, Erg25p, Erg27p, and Erg6p. We have described an interaction by coimmunoprecipitation between Erg28p and Erg25p (and Erg27p) in a previous study (12) in

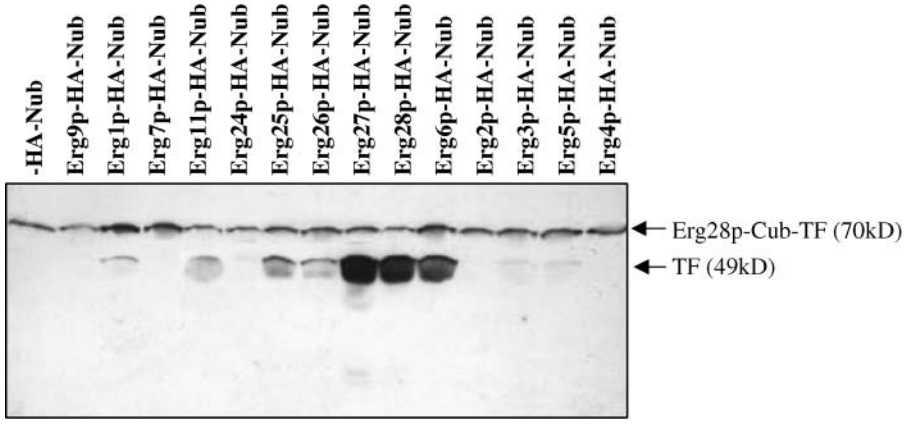

Fig. 5. Western blot from yeast cells containing the Erg28p-Cub-TF fusion protein with various ErgXp-HA-Nub preys. The intensity of the 70 kDa Erg28p-Cub-TF fusion protein band is compared with that of the released 49 kDa TF band. An interaction between Erg28-Cub-TF and the pNub control did not result in release of the TF. Immunoblots were obtained using anti-VP16 HRP.

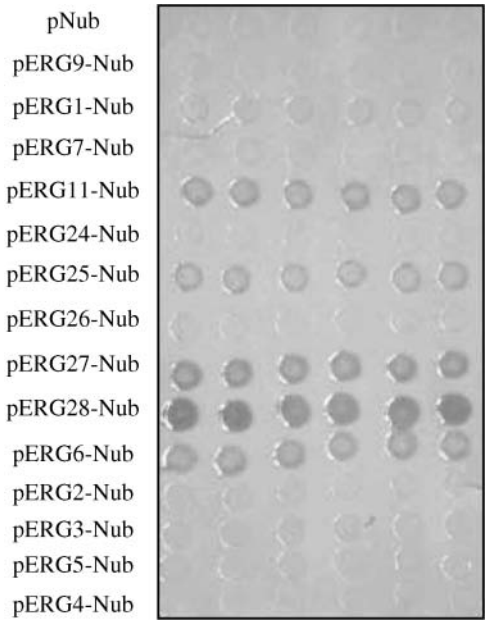

Fig. 6. β-Galactosidase assay using the *LacZ* reporter to assess interactions between Erg28p-Cub-TF and various ErgXp-HA-Nub preys. Six colony replicates containing bait and prey constructs were spotted onto sterilized nitrocellulose membranes, which were then overlaid onto CSM-Leu-Trp plates and grown for 48 h before assaying for β-galactosidase using 5-bromo-4-chloro-3-indolyl-β-D-galactoside (X-Gal) as substrate.

which we demonstrated that Erg28p tethers the C-4 demethylation enzymes to the ER. We now demonstrate that Erg11p is also a strong interaction partner. Erg28p also strongly interacts with Erg6p, which is considered to belong to the latter part of the sterol pathway. This interaction was also demonstrated previously by coimmunoprecipitation (13). A weaker interaction between Erg28p and Erg1p was also observed. Significantly, Erg28p interacts with itself.

Erg28p interacts with enzymes in ergosterol biosynthesis, as demonstrated by release of the TF

We compared the Erg28p-Cub-TF fusion protein and the truncated activated form of the TF fragment that is required to drive the *HIS3* and *LacZ* reporter genes (**Fig. 5**). The stronger the interaction between bait and prey, the greater the release of the truncated form of TF. For example, in Fig. 4, Erg28p and Erg27p appear to interact strongly, as indicated by growth on CSM-Leu-Trp-His + 5 mM 3-AT. In Fig. 5, we also observe a very significant level of the 49 kDa TF (released TF) relative to the 70 kDa nontruncated fusion protein. Other strong bait-prey interactions are observed between Erg28p and Erg6p, Erg28p and Erg25p, Erg28p and Erg11p, and Erg28p and itself. Weaker interactions can be seen between Erg28p and Erg26p and between Erg28p and Erg1p. A small 49 kDa band of released TF was also observed between Erg28p and Erg3p and between Erg28p and Erg5p, suggesting possible weak interactions between these proteins that were not apparent in the spot plate assay. All other bait-prey combinations (Erg28p-Erg9p, Erg28p-Erg7p, Erg28p-Erg24p, Erg28p-Erg2p, and Erg28-Erg4p) as well as the bait and control empty vectors showed no observable released TF.

Erg28p interacts with enzymes in ergosterol biosynthesis, as indicated by β-galactosidase filter assays

To corroborate these interactions, we performed filter assays using the *LacZ* reporter as a third assay. For each Erg28p interaction with pNub baits, six colonies for each prey were assayed (**Fig. 6**). Based on these results, the strongest interactions were between Erg28p and itself, Erg28p and Erg27p, Erg28p and Erg11p, Erg28p and Erg6p, and Erg28p and Erg25p, exactly confirming the spot plate results. Although interactions between Erg28p and Erg1p and between Erg28p and Erg26p appeared a little weaker than the first five interactions, there also appeared to be possible weak interactions between Erg28p and Erg3p and between Erg28p and Erg5p (based on a weak blue color). No other interactions appeared significantly different from the control.

Coimmunoprecipitation analyses confirm Erg11p and Erg1p as interaction partners of Erg28p

We previously demonstrated that Erg28p interacts with Erg25p, Erg26p, Erg27p, and Erg6p by coimmunoprecipitation. To confirm that Erg28p interacts with Erg11p and Erg1p (using a non-two-hybrid system), we coimmunoprecipitated Erg28p (using 500 μg of solubilized membrane extract) with various anti-Erg antibodies. **Figure 7A** demonstrates that anti-Erg1p, anti-Erg3p, anti-Erg6p, anti-Erg7p, anti-Erg11p, and anti-Erg25p all coimmunoprecipitate the Erg28p-Myc protein; the only exception is anti-Erg2p. However, because the signal strengths of the coimmunoprecipitation reactions are significantly different, attributable to the fact that each antibody recognizes different epitopes of the Erg28p partners, we cannot conclude anything regarding the strength of the interactions. As a control, the antibody to Lcb1p, which is a subunit of serine palmitoyltransferase (35), a ER membrane-associated protein required for sphingolipid synthesis, failed to coimmu-

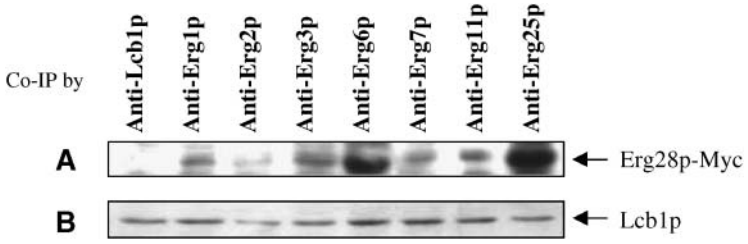

Fig. 7. Coimmunoprecipitation assays to demonstrate interactions between Erg28p and other ergosterol biosynthetic proteins. A: Erg28p-Myc was coimmunoprecipitated (Co-IP) by anti-Erg1p, anti-Erg3p, anti-Erg6p, anti-Erg7p, anti-Erg11p, and anti-Erg25p. B: Equivalent amounts of solubilized membrane protein were used for each coimmunoprecipitation reaction, as indicated by the anti-Lcb1p control immunoblot.

noprecipitate Erg28p (absence of any discernible band). Lcb1p was also used to demonstrate that equivalent amounts of solubilized membrane protein were used for each coimmunoprecipitation reaction. These results suggest that most ergosterol biosynthetic proteins may form a complex involving Erg28p.

DISCUSSION

The split-ubiquitin membrane YTH system is an invaluable tool in assessing membrane protein-protein interactions because it requires a direct interaction between two proteins. We expect that this system will give more definitive results than coimmunoprecipitation and affinity tag protocols, which require that two proteins can be precipitated in the same complex but not necessarily interact directly. In the case of ergosterol biosynthesis, virtually nothing is known regarding the topology of enzymes leading to ergosterol. This is equally true for sterol biosynthetic pathways in animals and plants. The yeast sterol pathway is completely delineated, yet no enzyme known to be a sterol binding protein designed to interact with other biosynthetic enzymes is known to exist, nor is there a known conserved amino acid motif common to sterol biosynthetic enzymes. The closest protein that may play a role in sterol synthesis found in all sterol biosynthetic pathways is Erg28p. Our previous results suggested that Erg28p coimmunoprecipitated with several other enzymes, but now we demonstrate direct protein-protein interactions. Previous reports also indicated that Erg28p is necessary for the proper functioning of the C-4 demethylation complex and that without this protein only one-third the amount of ergosterol is made. Additionally, in an *ERG28* null background, intermediates such as carboxylic acid and 3-keto sterols accumulate. Erg6p, which is found in both the ER and lipid droplets, is more widely dispersed into lipid droplets in the absence of Erg28p, further indicating the importance of this protein to the integrity of the sterol biosynthetic pathway. These studies confirm previous protein interactions and yet extend the role of Erg28p such that new interactions are observed, principally interactions with Erg1p and Erg11p, two very early enzymes in the pathway. We also demonstrate that Erg28p interacts with itself, suggesting that this protein may exist in multiple copies in the enzymatic complex to tether all of the enzymes into one or more complexes.

Erg28p is the first protein identified that is associated with the ergosterol biosynthetic pathway that does not appear to have an enzymatic function. The analyses presented here indicate that Erg28p strongly interacts with Erg27p, Erg25p, Erg6p, and Erg11p; less with Erg26p and Erg1p; and perhaps somewhat with Erg3p and Erg5p. Coimmunoprecipitation analyses with six different antibodies involving Erg1p, Erg3p, Erg6p, Erg7p, Erg11p, and Erg25p also indicated an interaction with Erg28p; thus, we suggest that a single complex of diverse enzymes may be held together by Erg28p and perhaps other uncharacterized proteins. These other proteins may have enzymatic as well as structural functions to facilitate the synthesis of sterols. An additional consideration is that at least three enzymes in yeast sterol biosynthesis (Erg1p, Erg6p, and Erg7p; reviewed in 36) and the ortholog of Erg26p in human cholesterol biosynthesis (37) have been demonstrated to also reside in lipid droplets. We have demonstrated previously that the amount of Erg6p in lipid droplets increases when Erg28p is not present, and it is possible that Erg28p with two transmembrane domains is required to tether these other enzymes to the ER. Sterol biosynthetic enzymes that reside in the ER but also may be found in lipid droplets all lack obvious transmembrane domains (36). A recent microarray study using mouse fibroblasts, in which the orthologous alleles of Erg26p (NSDHL sterol dehydrogenase) were deleted, resulted in a 2-fold increase of *Orf11* (the mouse ortholog of Erg28p). Among the 22,000 genes screened, the expression of only 4 genes was significantly higher than that of *Orf11* (38). Because Erg28p is broadly conserved in fungi, plants, and animals, it may be responsible for the integrity of the ergosterol biosynthetic enzyme complex.

The authors thank Dr. Igor Stagljar for his generous gifts of bait and prey vectors and Dr. Safia Thaminy for extensive discussions regarding the yeast split-ubiquitin two-hybrid system. The authors thank Drs. Friederike Turnowsky and Guenther Daum for antibodies to ergosterol biosynthetic proteins and Sung Wu, Alex Pierson, and Aaron Sturm, who provided technical expertise in this study. This investigation was supported by National Institutes of Health Grant GM-62104 to M.B.

REFERENCES

1. Faust, J. R., J. M. Trazaskos, and J. L. Gaylor. 1988. Cholesterol biosynthesis. *In* Biology of Cholesterol. P. Yeagle, editor. CRC Press, Boca-Raton, FL. 19–39.
2. Benveniste, P. 1986. Sterol biosynthesis. *Annu. Rev. Plant Physiol.* **55:** 429–457.
3. Lees, N. D., and M. Bard. 2003. Sterol biochemistry and regulation in the yeast *Saccharomyces cerevisiae*. *In* Topics in Current Genetics. Vol. 6. G. Daum, editor. Springer-Verlag, Heidelberg, Germany. 213–240.
4. Sturley, S. L. 2000. Conservation of eukaryotic sterol homeostasis: new insights from studies in budding yeast. *Biochim. Biophys. Acta.* **1529:** 155–163.
5. Yang, H., M. Bard, D. A. Bruner, A. Gleeson, R. J. Deckelbaum, G. Aljinovic, T. M. Pohl, R. Rothstein, and S. L. Sturley. 1996. Sterol esterification in yeast: a two-gene process. *Science.* **272:** 1353–1356.
6. Parks, L. W., C. D. Bottema, R. J. Rodriguez, and T. A. Lewis. 1985. Yeast sterols: yeast mutants as tools for the study of sterol metabolism. *Methods Enzymol.* **111:** 333–346.
7. Yang, H., D. Cromley, H. Wang, J. T. Billheimer, and S. L. Sturley. 1997. Functional expression of a cDNA to human acyl-coenzyme A:cholesterol acyltransferase in yeast. Species-dependent substrate specificity and inhibitor sensitivity. *J. Biol. Chem.* **14:** 3980–3985.
8. Hughes, T. R., M. J. Marton, A. R. Jones, C. J. Roberts, R. Stoughton, C. D. Armour, H. A. Bennett, E. Coffey, H. Dai, Y. D. He, et al. 2000. Functional discovery via a compendium of expression profiles. *Cell.* **102:** 109–126.
9. Gachotte, D., J. Eckstein, R. Barbuch, T. Hughes, C. Roberts, and M. Bard. 2001. A novel gene conserved from yeast to humans is involved in sterol biosynthesis. *J. Lipid Res.* **42:** 150–154.
10. Ottolenghi, C., I. Daizadeh, A. Ju, S. Kossida, G. Renault, M. Jacquet, M. A. Fellous, W. Gilbert, and R. Veitia. 2000. The genomic

structure of c14orf1 is conserved across eukarya. *Mamm. Genome.* **11:** 786–788.
11. Veitia, R., C. Ottolenghi, M. C. Bissery, and A. Fellous. 1999. A novel human gene, encoding a potential membrane protein conserved from yeast to man, is strongly expressed in testis and cancer cell lines. *Cytogenet. Cell Genet.* **85:** 217–220.
12. Mo, C., M. Valachovic, S. K. Randall, J. T. Nickels, and M. Bard. 2002. Protein-protein interactions among C-4 demethylation enzymes involved in yeast sterol biosynthesis. *Proc. Natl. Acad. Sci. USA.* **99:** 9739–9744.
13. Mo, C., M. Valachovic, and M. Bard. 2004. The *ERG28*-encoded protein, Erg28p, interacts with both the sterol C-4 demethylation enzyme complex as well as the late biosynthetic protein, the C-24 sterol methyltransferase (Erg6p). *Biochim. Biophys. Acta.* **1686:** 30–36.
14. Thaminy, S., D. Auerbach, A. Arnoldo, and I. Stagljar. 2003. Identification of novel ErbB3-interacting factors using the split-ubiquitin membrane yeast two-hybrid system. *Genome Res.* **13:** 1744–1753.
15. Gavin, A. C., M. Bosche, R. Krause, P. Grandi, M. Marzioch, A. Bauer, J. Schultz, J. M. Rick, M.A. Michon, C. M. Cruciat, et al. 2002. Functional organization of the yeast proteome by systematic analysis of protein complexes. *Nature.* **415:** 141–147.
16. Ho, Y., A. Gruhler, A. Heilbut, G. D. Bader, L. Moore, S. L. Adams, A. Millar, P. Taylor, K. Bennett, K. Boutilier, et al. 2002. Systematic identification of protein complexes in *Saccharomyces cerevisiae* by mass spectrometry. *Nature.* **415:** 180–183.
17. Fields, S., and O. Song. 1989. A novel genetic system to detect protein-protein interactions. *Nature.* **340:** 245–246.
18. Broder, Y. C., S. Katz, and A. Aronheim. 1998. The ras recruitment system, a novel approach to the study of protein-protein interactions. *Curr. Biol.* **8:** 1121–1124.
19. Hubsman, M., G. Yudkovsky, and A. Aronheim. 2001. A novel approach for the identification of protein-protein interaction with integral membrane proteins. *Nucleic Acids Res.* **29:** E18.
20. Ehrhard, K. N., J. J. Jacoby, X. Y. Fu, R. Jahn, and H. G. Dohlman. 2000. Use of G-protein fusions to monitor integral membrane protein-protein interactions in yeast. *Nat. Biotechnol.* **18:** 1075–1079.
21. Johnsson, N., and A. Varshavsky. 1994. Split ubiquitin as a sensor of protein interactions in vivo. *Proc. Natl. Acad. Sci. USA.* **91:** 10340–10344.
22. Wilkinson, K. D. 2004. Ubiquitin: a Nobel protein. *Cell.* **119:** 741–745.
23. Miller, J., and I. Stagljar. 2004. Using the yeast two-hybrid system to identify interacting proteins. *Methods Mol. Biol.* **261:** 247–262.
24. Wang, B., J. Pelletier, M. J. Massaad, A. Herscovics, and G. C. Shore. 2004. The yeast split-ubiquitin membrane protein two-hybrid screen identifies BAP31 as a regulator of the turnover of endoplasmic reticulum-associated protein tyrosine phosphatase-like B. *Mol. Cell. Biol.* **24:** 2767–2778.
25. Pandey, S., and S. M. Assmann. 2004. The *Arabidopsis* putative G protein-coupled receptor GCR1 interacts with the G protein alpha subunit GPA1 and regulates abscisic acid signaling. *Plant Cell.* **16:** 1616–1632.
26. Obrdlik, P., M. El-Bakkoury, T. Hamacher, C. Cappellaro, C. Vilarino, C. Fleischer, H. Ellerbrok, R. Kamuzinzi, V. Ledent, D. Blaudez, et al. 2004. K^+ channel interactions detected by a genetic system optimized for systematic studies of membrane protein interactions. *Proc. Natl. Acad. Sci. USA.* **101:** 12242–12247.
27. Muhlrad, D., R. Hunter, and R. Parker. 1992. A rapid method for localized mutagenesis of yeast genes. *Yeast.* **8:** 79–82.
28. Sikorski, R. S., and J. D. Boeke. 1991. *In vitro* mutagenesis and plasmid shuffling—from cloned gene to mutant yeast. *Methods Enzymol.* **194:** 302–318.
29. Sambrook, J., E. F. Fritsch, and T. Maniatis. 1989. Molecular Cloning: A Laboratory Manual. Cold Spring Harbor Laboratory Press, Plainview, NY.
30. Burke, D., D. Dawson, and T. Stearns. 2000. Methods in Yeast Genetics. Cold Spring Harbor Laboratory Press, Plainview, NY.
31. Leber, R., K. Landl, E. Zinser, H. Ahorn, A. Spok, S. D. Kohlwein, F. Turnowsky, and G. Daum. 1998. Erg1p, in yeast reflects a relationship between the endoplasmic reticulum and lipid particles. *Mol. Biol. Cell.* **9:** 375–386.
32. Leber, R., E. Zinser, G. Zellnig, F. Paltauf, and G. Daum. 1994. Characterization of lipid particles of the yeast, *Saccharomyces cerevisiae. Yeast.* **10:** 1421–1428.
33. Milla, P., K. Athenstaedt, F. Viola, S. Oliaro-Bosso, S. D. Kohlwein, G. Daum, and G. Balliano. 2002. Yeast oxidosqualene cyclase (Erg7p) is a major component of lipid particles. *J. Biol. Chem.* **277:** 2406–2412.
34. Li, J., and J. Kaplan. 1996. Characterization of yeast methyl sterol oxidase (*ERG25*) and identification of a human homologue. *J. Biol. Chem.* **271:** 16927–16933.
35. Gable, K., H. Slife, D. Bacikova, E. Monaghan, and T. M. Dunn. 2000. Tsc3p is an 80-amino acid protein associated with serine palmitoyltransferase and required for optimal enzyme activity. *J. Biol. Chem.* **275:** 7597–7603.
36. Mullner, H., D. Zweytick, R. Leber, F. Turnowsky, and G. Daum. 2004. Targeting of proteins involved in sterol biosynthesis to lipid particles of the yeast *Saccharomyces cerevisiae. Biochim. Biophys. Acta.* **1663:** 9–13.
37. Caldas, H., and G. E. Herman. 2003. NSDHL, an enzyme involved in cholesterol biosynthesis, traffics through the Golgi and accumulates on ER membranes and on the surface of lipid droplets. *Hum. Mol. Genet.* **12:** 2981–2991.
38. Cunningham, D., D. Swartzlander, S. Liyanarachchi, R. V. Davuluri, and G. E. Herman. 2005. Changes in gene expression associated with loss of function of the NSDHL sterol dehydrogenase in mouse embryonic fibroblasts. *J. Lipid Res.* **46:** 1150–1162.