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Tritium Suicide Selection Identifies Proteins Involved in the Uptake and Intracellular Transport of Sterols in *Saccharomyces cerevisiae*▽

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Sterol transport between the plasma membrane (PM) and the endoplasmic reticulum (ER) occurs by a nonvesicular mechanism that is poorly understood. To identify proteins required for this process, we isolated *Saccharomyces cerevisiae* mutants with defects in sterol transport. We used *Upc2-1* cells that have the ability to take up sterols under aerobic conditions and exploited the observation that intracellular accumulation of exogenously supplied [^{3}H]cholesterol in the form of [^{3}H]cholesteryl ester requires an intact PM-ER sterol transport pathway. *Upc2-1* cells were mutagenized using a transposon library, incubated with [^{3}H]cholesterol, and subjected to tritium suicide selection to isolate mutants with a decreased ability to accumulate [^{3}H]cholesterol. Many of the mutants had defects in the expression and trafficking of Aus1 and Pdr11, PM-localized ABC transporters that are required for sterol uptake. Through characterization of one of the mutants, a new role was uncovered for the transcription factor Mot3 in controlling expression of Aus1 and Pdr11. A number of mutants had transposon insertions in the uncharacterized Ydr051c gene, which we now refer to as *DET1* (*d*ecreased *e*rgosterol *t*ransport). These mutants expressed Aus1 and Pdr11 normally but were severely defective in the ability to accumulate exogenously supplied cholesterol. The transport of newly synthesized sterols from the ER to the PM was also defective in *det1*Δ cells. These data indicate that the cytoplasmic protein encoded by *DET1* is involved in intracellular sterol transport.

Sterols, such as cholesterol in mammalian cells and ergosterol in yeast, are important constituents of all eukaryotic cells (33, 34, 36, 48). They are synthesized in the endoplasmic reticulum (ER) but enriched ~5- to 10-fold in the plasma membrane (PM) (30, 46); in the yeast *Saccharomyces cerevisiae*, ~70% of total cellular ergosterol is located in the PM (3, 49). The mechanism by which sterols are transported between the ER and PM is poorly understood. Since the spontaneous diffusion of hydrophobic sterol molecules through an aqueous environment is thermodynamically unfavorable, cells must have a mechanism to transport sterols between membranes (33, 38). Studies with yeast and mammalian cells indicate that sterol transport between the ER and the PM does not depend on a functional vesicular transport pathway but requires ATP and is blocked at low temperatures (3, 18, 24, 29, 32, 45, 50).

Proteins required for nonvesicular sterol transport between the ER and PM have not been identified convincingly (33, 38). There are numerous known sterol-binding proteins that could function in nonvesicular sterol transport, either directly as carriers or in an accessory role. Proteins with lipid-binding START domains (2) are attractive candidates for cytoplasmic sterol carriers. One member of the START family, the ceramide transporter CERT, was shown to catalyze the ATP-dependent transport of ceramide from the ER to the Golgi apparatus (14, 15). Recent structural analyses indicate that CERT specifically sequesters ceramide in a hydrophobic binding pocket to shield the lipid from the aqueous environment during transit through the cytoplasm (28). Targeting motifs within CERT enable it to engage the ER and Golgi apparatus specifically, providing a mechanism to restrict the ceramide transport pathway to these two organelles. Yeast cells lack proteins with START domains but contain other proteins with lipid-binding motifs. The seven oxysterol binding protein homologs, or Osh proteins (4), are prominent examples of soluble yeast proteins with putative lipid-binding domains. One of the Osh proteins, Osh4/Kes1, has been analyzed by X-ray crystallography in complex with different sterols (25) and shown to transport sterols between donor and acceptor vesicles in vitro (39). Elimination of the Osh proteins in particular combinations influences sterol transport in vivo (39, 49). However, it remains to be seen whether the true function of the Osh proteins is to transport sterols or to act in other ways, for example, as sterol sensors in regulating sterol distribution within the cell (10, 55).

A recent study attempted to identify proteins involved in sterol transport by using a visual screen for the transport-dependent accumulation of a fluorescent analog of cholesterol (40). The screen was limited to genes required for anaerobic growth. While the study discovered an unexpected role for mitochondria in PM-ER sterol transport, it did not identify a sterol transporter. Here we report a tritium suicide selection strategy designed to identify yeast mutants with defects in sterol transport, thus providing an unbiased way to identify components of the nonvesicular sterol transport system. The selection operates on the premise that cells that are unaffected in a function/pathway of interest will accumulate large amounts of a tritiated substrate, whereas those with defects in that function or pathway will not. When a mutagenized culture of cells is incubated with an appropriate tritium-labeled substrate and stored at −80°C, cells that efficiently accumulate the radiolabeled probe undergo radiation-induced DNA damage and die, leaving behind an enriched pool of mutants.

We exploited the transport-dependent accumulation of

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TABLE 1. Strains used in this study

Strain	Genotype	Source
WPY361	*MAT***a** *upc2-1 ura3-1 his3-11,15 leu2-3,112 trp1-1*	32
DSY104	WPY361/p*MOT3-URA3*	This study
C209	WPY361 *det1*::mTn*3*	This study
BY4741	*MAT***a** *his3-1 leu2-0 ura2-6 met15-1*	54; EUROSCARF
*det1*Δ	BY4741 *det1*::*KanMX4*	54; EUROSCARF

exogenous [^{3}H]cholesterol in *Upc2-1* cells mutagenized with a transposon library, in conjunction with tritium suicide selection, to uncover mutants defective in the incorporation of exogenous sterols. Analysis of the survivors uncovered numerous genes involved in the uptake and transport of exogenous sterols. We identified the transcription factor Mot3 as a repressor of *AUS1* and *PDR11*, genes that encode PM-localized ATP-binding cassette (ABC) transporters involved in sterol uptake. We also identified the previously uncharacterized open reading frame (ORF) Ydr051c, which we now refer to as *DET1* (*d*ecreased *e*rgosterol *t*ransport). Transport of sterols between the ER and PM was significantly reduced in *det1*Δ cells, indicating that the cytoplasmic protein encoded by *DET1* is involved in intracellular sterol transport.

MATERIALS AND METHODS

Materials. All radiolabeled compounds were purchased from American Radiolabeled Chemicals (St. Louis, MO). Methyl-β-cyclodextrin (MβCD), ergosterol, cholesterol, Tween 80, FM4-64, calcofluor white, Nile red, and sodium azide were purchased from Sigma-Aldrich (St. Louis, MO). RenoCal-76 was purchased from Bracco Diagnostics (Princeton, NJ). Growth media and reagents were purchased from EMD Chemicals Inc. (Darmstadt, Germany). Antibodies against Sec61, Vph1, and Gas1 were gifts from T. Rapoport (Harvard Medical School, Boston, MA), P. Kane (SUNY-Upstate Medical Center, Syracuse, NY), and A. Conzelmann (University of Fribourg, Switzerland), respectively. Antibodies against Pep12 were obtained from Molecular Probes. All other reagents were purchased from VWR (Westchester, PA).

Growth conditions, cloning, and common techniques. Strains used in this study are listed in Table 1. Unless otherwise stated, yeast cultures were grown in a rich medium (YPD; 1% [wt/vol] yeast extract, 2% [wt/vol] peptone, 2% [wt/vol] glucose) with shaking at 250 rpm at 25°C. Strains carrying plasmids were grown under selective conditions in synthetic media. Growth of cells in suspension was monitored by the optical density at 600 nm (OD_{600}). DNA cloning and bacterial and yeast transformations were carried out using standard techniques (13, 44). To clone *MOT3*, DNA was amplified from WPY361 genomic DNA using PCR and primers with engineered restriction sites for XhoI and HindIII. Primers were designed to include the entire ORFs and ~1,000 bp of the 5′ untranslated region (UTR). The digested product was ligated into pRS316 and confirmed by sequencing. Aus1-GFP and Pdr11-GFP were expressed as C-terminal fusion proteins under the control of their endogenous promoters from the plasmids pWP1220 and pWP1251, respectively (32).

Tritium suicide selection to isolate yeast mutants in intracellular sterol transport. We adapted the tritium suicide selection procedure described by Huffaker and Robbins (21, 22). The mTn-LacZ/Leu2 transposon library described by Burns et al. (6) and Ross-Macdonald et al. (42) was used to mutagenize WPY361 (*Upc2-1*) cells. Approximately 30,000 combined transformants generated from library pools 24 and 28 were used for selection (~15,000 from each pool). Transformants from each pool were subjected to selection separately. Suspensions of the transformants obtained by recovering the colonies in sterile water were used to inoculate overnight cultures. The following day, the cultures were diluted to 0.25 OD_{600} unit/ml in 50 ml and allowed to undergo one doubling. Twenty-five OD_{600} units of these cultures was harvested, resuspended in 250 μl YPD, and incubated at 37°C for 15 min. A total of 10 OD_{600} units (100 μl) was collected, resuspended in YPD containing 1 mCi of [^{3}H]cholesterol in 4% (vol/vol) Tween 80–ethanol (1:1 [vol/vol]), and vortexed vigorously. A parallel control sample of cells was resuspended similarly, except that unlabeled cholesterol was used. The samples were incubated at 37°C for 1 h, after which the cells were collected, washed thrice with ice-cold water, and resuspended in 1 ml of ice-cold water. Aliquots of cells were removed to determine cell number (by plating serial dilutions) as well as the extent of [^{3}H]cholesterol uptake and conversion to cholesteryl ester (see below). The remaining cells were diluted with an equal volume of 20% (vol/vol) glycerol, divided into 50-μl aliquots, and stored at −80°C. Aliquots were periodically removed, and serial dilutions were plated to determine cell survival. When the survival rate approached a minimum, the remaining aliquots were removed and plated on YPD at a density of ~100 colonies per plate. Cholesterol uptake on these plates was analyzed as described below. To ensure that mutants with temperature-sensitive defects were not missed, the survivors were assayed for the ability to incorporate sterols at 37°C. The site of transposon insertion was determined using EcoRI to digest genomic DNA and standard protocols associated with the library (6). The recovered mutants discussed here were all verified by Southern blot analysis or by recreating the disruption in *Upc2-1* to ensure that the defects observed were caused by a single insertion in the indicated ORF. The probe for Southern blot analysis of the EcoRI-digested genomic DNA was created using PCR and the primers ACC TGC GTT TCA CCC TGC CAT AAA GA and ACC CAA CTT AAT CGC CTT GCA GCA CA, which recognize a portion of the *lacZ* gene in the insert backbone. This PCR product was labeled using random primer extension and [^{32}P]CTP.

Uptake of cholesterol by yeast on plates. To assess cholesterol uptake of multiple colonies simultaneously, the protocol of Lewis et al. (31) was used. Briefly, colonies were spotted onto YPD plates, grown for 2 days at room temperature, and then replica plated onto a YPD plate overlaid with a nitrocellulose disc. After allowing the cells to grow for 2 days at room temperature, the disc was overlaid onto a YPD plate containing 1% (vol/vol) Tween 80–ethanol (1:1 [vol/vol]) and 0.5 μCi/ml [^{14}C]cholesterol. After 4 h, the disc was removed and treated to remove nonspecifically bound [^{14}C]cholesterol. This was accomplished by first placing the disc with the colony side up on Whatman paper soaked with 0.5% (wt/vol) NP-40 and then on dry paper towels. After repeating the treatment thrice, the colonies on the disc were transferred to Whatman paper by pressing the two together in a sandwich of paper towels. The disc was removed, and the Whatman paper was wrapped in plastic wrap and exposed to a phosphorimager plate overnight to visualize [^{14}C]cholesterol uptake by the individual colonies.

Uptake of cholesterol by cells in suspension. Measurement of cholesterol uptake by suspension-grown yeast cells was done by a procedure adapted from the work of Li and Prinz (32). Briefly, overnight cultures were diluted to 0.5 OD_{600} unit/ml in 2 ml of YPD. After 2 h (roughly one doubling period), the cultures were shifted to 37°C and preincubated for 15 min before adding a mixture of cholesterol and [^{3}H]cholesterol in 1:1 (vol/vol) Tween 80–ethanol. The final concentration (each) of Tween 80 and ethanol was 0.5% (vol/vol), and the final concentration of cholesterol was 1 μM, with 10 μCi of [^{3}H]cholesterol. After incubation at 37°C for 1 h, 1.5 ml of each culture was removed and collected. Cell pellets were washed three times with 1 ml ice-cold 0.5% (vol/vol) Tween 80 in water and then resuspended in 200 μl ice-cold water. Acid-washed glass beads (200 μl) were added, and the cells were disrupted by being vortexed (six times for 30 s each, interspersed with 30-s cooling periods on ice). The resulting cell homogenate was collected, and the beads were washed with 800 μl of water. The homogenate and wash were combined and then extracted with 3 ml of hexanes-isopropanol (3:2 [vol/vol]). After thorough mixing of the sample, the organic phase was allowed to separate and then recovered and replaced with 2 ml of hexanes. The combined organic phases were dried and dissolved in 100 μl of chloroform-methanol (1:1 [vol/vol]), from which 10 μl was removed for scintillation counting to determine the total amount of radioactivity taken up by the cells and 35 μl was analyzed by thin-layer chromatography (TLC) to quantify the amount of label incorporated into cholesteryl esters and free cholesterol. For TLC, the samples were spotted onto silica gel 60 plates and resolved using hexanes-diethyl ether-acetic acid (70:30:1 [vol/vol/vol]) as the solvent system. Free cholesterol and cholesteryl esters had R_f values of 0.15 and 0.75, respectively. The relative amounts of radioactivity in the peaks were quantitated using a Berthold LB2842 TLC scanner.

Transport of ergosterol from the ER to the PM. Transport assays were done as described by Baumann et al. (3) at 37°C with a 15-min preincubation in prewarmed medium. Briefly, cells were pulse labeled with [^{3}H-methyl]methionine and then chased. Aliquots were removed at different time points and incubated with MβCD on ice to extract PM ergosterol. Ergosterol in the MβCD extract, as well as that in the cell sample, was quantitated by organic solvent extraction and high-performance liquid chromatography (HPLC) analysis. Fractions were collected and taken for scintillation counting to determine the amount

TABLE 2. Primers used in this study

Primer	Sequence (5′ to 3′)
Mot3 f1 XhoI	ACTTCTCGAGACGGCGTGCTAGGTGCATTTACTTCA
Mot3 r1 HindIII	CTGTAAGCTTCTTAAATGAGTGGGAAGGGATATTTTGTGTGTC
RT Act1 f1	CATGCCAGTAACCTCAAATGAGCC
RT Act1 r1	CGAGATGCTCTGCAAAGAAGGCAA
RT Aus1 f1	TTCCTAGACGAACCAACCTCTG
RT Aus1 r1	CCGAGGAGTTACTGTTACCTACTG
RT Pdr11 f1	GCGCTCTTCAAAGGTCTATTCC
RT Pdr11 r1	CACCACGAACGTAGTCATTACC
RT Mot3 f1	AGGACGACGTTATGGTGAACTCTG
RT Mot3 r1	CCGCAGTAAACTGGTTTGGGTGAA

of [^{3}H]ergosterol, whereas bulk ergosterol was quantitated by an in-line UV detector set at 280 nm. The specific radioactivity (SR) of [^{3}H]ergosterol was determined in each case, and the SR of the MβCD sample was normalized to the SR of the cell sample to determine relative specific radioactivity (RSR) (RSR = $SR_{M\beta CD\ extract}/SR_{cell}$). The RSR directly compares the specific radioactivity of PM-localized ergosterol extracted by MβCD with that of total cellular ergosterol; plots of RSR versus time provide a measure of the rate at which ergosterol is transported from the ER to the PM.

Subcellular distribution of ergosterol. Total membranes from ~20 OD_{600} units of cells (grown to an OD_{600} of ~1 at room temperature and then incubated for 15 min at 37°C prior to homogenization) were fractionated on step gradients of RenoCal-76 exactly as described previously (3, 7, 32). Sterol content in the pooled intracellular and PM fractions (identified by sodium dodecyl sulfate-polyacrylamide gel electrophoresis immunoblotting using organelle-specific antibodies) was determined using reverse-phase HPLC as described above. Sterol/phospholipid ratios were determined from lipid extracts obtained by hot ethanol extraction (16) and desalted by *n*-butanol–water partitioning. Sterol was quantitated using HPLC as described below, and lipid phosphorus was quantitated exactly as described previously (43).

QPCR. Overnight cultures were diluted to 0.5 OD_{600} unit/ml and incubated for 2 h at 25°C. Cells were harvested, transferred to prewarmed YPD, and incubated at 37°C for 45 min. RNA was isolated from 2 OD_{600} units of cells by use of an RNeasy Mini kit (Qiagen), following the protocol for mechanical disruption of cells using glass beads. cDNA was prepared using Brilliant SYBR green QRT-PCR AffinityScript two-step master mix (Stratagene) following the provided protocol. Quantitative PCR (QPCR) amplification of cDNA was performed using the same kit according to the manufacturer's protocol. The primers used are listed in Table 2. QPCR was carried out using Stratagene's MX3005P and MX4000 QPCR systems. The thermal profile was as follows: 10 min at 95°C and 40 cycles of 30 s at 95°C, 1 min at 50°C, and 30 s at 72°C. Fluorescence readings were taken at the end of each 50°C annealing cycle. A melting curve from 50°C to 95°C was performed in each experiment after the last cycle to determine the specificity of the primers for a single sequence. Data were analyzed using Stratagene's MxPro software and normalized to *ACT1* mRNA levels.

Microscopy. For examination of the cellular distribution of Aus1-GFP and Pdr11-GFP, cells from exponentially growing cultures carrying the indicated green fluorescent protein (GFP) constructs were grown in selective media lacking the appropriate nutrient and visualized on coverslip dishes at room temperature in the same media. For Nile red staining, exponentially growing cultures were harvested, washed once, and resuspended in 1 ml of water. Two microliters of 1-mg/ml Nile red in acetone was added and incubated for 5 min at room temperature. Cells were then collected, washed twice with water, and resuspended to 1.5 OD units/ml in water. One hundred microliters of cells was allowed to settle on coverslip dishes before visualization.

All images were acquired at a magnification of ×63, using an oil immersion lens on a Zeiss Axiovert 200 wide-field microscope equipped with a MicroMax interline charge-coupled device camera driven by MetaMorph Imaging System software (Universal Imaging Corp.). Images were taken with the same exposure times and processed to maintain intensity differences using Adobe Photoshop CS2 (Adobe Systems, Mountain View, CA).

RESULTS

Suicide selection of sterol transport mutants. Under aerobic conditions, wild-type *S. cerevisiae* cells do not take up exogenous sterol into their membranes (a phenomenon termed aerobic sterol exclusion) and instead synthesize their own (9). However, the WPY361 strain (Table 1), which has a gain-of-function point mutation in the transcription factor Upc2, is able to incorporate sterols from the growth medium. WPY361 cells (henceforth referred to as *Upc2-1* cells) express a number of proteins that are not normally expressed under aerobic conditions. These proteins include the cell wall mannoprotein Dan1 and two ABC transporters, Aus1 and Pdr11, that have been shown to be involved in the uptake of exogenous sterols (1, 53).

When *Upc2-1* cells are supplied with [^{3}H]cholesterol, they convert it efficiently to [^{3}H]cholesteryl esters that accumulate in lipid droplets (32). It is likely that the exogenously supplied sterol is first incorporated into the PM via a mechanism involving Dan1, Aus1, and Pdr11 and then transported to the ER, where it is esterified by the ER-localized yeast acyl-coenzyme A cholesterol acyl transferases Are1 and Are2 (56) (Fig. 1A). The cellular accumulation of cholesterol is strongly dependent on esterification, such that in the absence of Are1 and Are2, the total amount of internalized cholesterol is low (32). In contrast to cholesterol, exogenously supplied ergosterol is poorly esterified since it remains localized to the PM (see Discussion) (32).

Because the accumulation of cholesterol requires its transport from the PM to the ER, we chose a tritium suicide selection strategy to isolate mutants with defects in sterol transport. *Upc2-1* cells were mutagenized with two different pools (pools 24 and 28) of the mTn*3* LacZ/Leu2 transposon library (42) to create insertions in genomic DNA and to disrupt numerous different ORFs. Roughly 15,000 colonies from each library pool were collected and subjected to suicide selection. An exponentially growing culture was loaded with [^{3}H]cholesterol for 60 min at 37°C, during which time the culture internalized ~10 cpm/cell. The culture was washed, divided into aliquots, and frozen at −80°C. A parallel sample was loaded with unlabeled cholesterol and treated similarly. During cold storage, cells that internalize a significant amount of the [^{3}H]cholesterol are predicted to accumulate radiation-induced DNA damage and die. In contrast, mutants with defects in sterol accumulation, including cells with defects in PM-ER sterol transport, are likely to survive. To check the survival of the culture, aliquots of cells were removed periodically, and the number of survivors was determined by plating serial dilutions. The proportion of viable cells in the control culture remained relatively constant, at ~30% (Fig. 1B). As predicted, the [^{3}H]cholesterol-loaded sample showed a dramatic decrease in survival, with only ~0.05% of the cells remaining after 80 days (Fig. 1B). At this point, the remaining aliquots of the loaded culture were removed from the freezer and plated on YPD; 4,000 survivors were recovered from an initial load of 8×10^6 cells.

The survivors were screened for the ability to incorporate exogenous cholesterol. Colonies were transferred from solid medium to a nitrocellulose disc that was then overlaid onto a [^{14}C]cholesterol-containing plate; after a period of incubation,

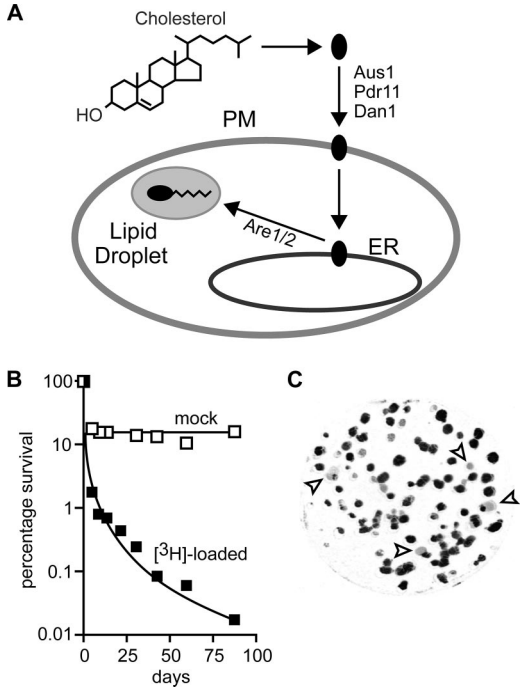

FIG. 1. [^{3}H]cholesterol suicide selection. (A) Schematic illustration showing the uptake, PM-ER transport, and accumulation of cholesterol in the form of cholesteryl ester in a *Upc2-1* cell. (B) Killing curve. *Upc2-1* cells were mutagenized with a transposon library. After outgrowth, 8×10^6 cells were either loaded with [^{3}H]cholesterol or mock treated with nonlabeled cholesterol. Aliquots of the cells were frozen and stored at −80°C. At the indicated times, aliquots were removed from the freezer, thawed, and plated to determine the number of surviving colonies. The graph shows the percent survival of ^{3}H-loaded and mock-treated cells after storage at −80°C. (C) Colonies that survived the suicide selection were gridded, replica plated onto nitrocellulose paper, and overlaid on agar containing [^{14}C]cholesterol. After incubation, colony-associated radioactivity was visualized with a phosphorimager. Open arrowheads indicate examples of colonies with decreased uptake that were picked for further study.

the disc was removed and washed, and the extent of [^{14}C]cholesterol uptake was visualized with a phosphorimager (Fig. 1C). Based on this assay, a total of 364 mutants from both pools of the mTn*3* library had a defect in sterol uptake, as defined by a decreased ability to incorporate sterols at 37°C, although the mutants varied dramatically in the extent of the defect. The site of transposon insertion in the mutants was identified as described previously (42). The mutant collection was screened until multiple hits were recovered in some of the different ORFs. We used Southern analysis (or recreation of the gene disruption, if necessary) to verify that the defect in sterol accumulation in the mutants was due to the disruption of a single gene. Through this process, we identified 23 nonessential genes with a putative role in sterol transport (Table 3). Disruptions in *UPC2*, *RDN25-1*, *TVP18*, and the YDR051c gene were observed at greater frequencies than disruptions in the other ORFs listed in Table 3. Because Upc2-1 is critical for sterol uptake in this strain, the recovery of insertions that disrupted the *UPC2* locus was expected and confirmed the validity of our approach.

Suicide selection survivors show reduced incorporation of exogenous sterols. The 23 genes that we identified in the suicide selection fell into a number of broad categories, and many did not have an assigned function according to the *Saccharomyces* Genome Database (http://www.yeastgenome.org). In our initial analyses, we chose to focus on the 14 genes in the categories "regulation of protein signaling" and "unknown" listed in Table 3 since, based on available information, they appeared most likely to be involved in an aspect of sterol transport.

To confirm that the 14 mutants corresponding to these two categories were defective in accumulating exogenously supplied sterol, we determined cholesterol uptake using suspension-grown cells. This provides a quantitative complement to the more qualitative filter uptake assay used in our initial screen. Exponentially growing cultures of the 14 mutants were incubated for 60 min with 1 μM cholesterol and a trace amount of [^{3}H]cholesterol. Relative to the parent *Upc2-1* strain, all mutants had a decreased ability to accumulate exogenous sterols (Fig. 2A). Six of the mutants, those with inserts in *AIM44*, *HOF1*, *RAM1*, *TVP18*, *SCY1*, and the YDR051c gene (which we henceforth refer to as *DET1* [*d*ecreased *e*rgosterol *t*ransport]), had significantly reduced accumulation that approached the level of a wild-type strain (BY4741) that lacks the ability to take up exogenous sterols because of aerobic sterol exclusion. Of the small amount of sterol that was taken up by these six mutants, a significant portion (50 to 90%) in all strains was esterified to cholesteryl ester, as determined by separating and quantitating the incorporated [^{3}H]cholesterol by TLC (Fig. 2B). These data suggest that the poor accumulation of [^{3}H]cholesterol in the six mutants was not due to defective esterification.

Mot3 regulates the expression of Aus1 and Pdr11. We repeatedly recovered mutants with transposon insertions in *TVP18*. Although *TVP18* encodes a Golgi membrane protein of unknown function (Table 3) that could potentially play a role in sterol transport and accumulation, we considered a different possibility. We noticed that the *TVP18* locus is located adjacent to the *MOT3* locus in the yeast genome. Mot3 is a transcription factor that is known to repress numerous anaerobic/hypoxic genes under aerobic conditions (19, 26, 27, 47). We hypothesized that insertion of a transposon into the *TVP18* locus could have an indirect effect on sterol uptake by increasing the transcription of *MOT3*. To test this possibility, we used reverse transcription-QPCR to quantitate levels of *MOT3*, *AUS1*, and *PDR11* mRNAs in the *tvp18*::mTn*3* mutant as well as the *Upc2-1* strain. Figure 3 shows that the level of *MOT3* mRNA was significantly increased in the *tvp18*::mTn*3* mutant compared to the *Upc2-1* cells, while *AUS1* and *PDR11* transcript levels were reduced. To investigate this further, we overexpressed Mot3 in the *Upc2-1* strain; as expected, *MOT3* mRNA levels were increased, while the levels of *AUS1* and *PDR11* were reduced. Consistent with the reduction in *AUS1* and *PDR11* transcript levels, exogenous sterol incorporation in the Mot3-overexpressing strain was reduced 60% ± 2% (at room temperature; incorporation was reduced ~70% at 37°C) relative to that in the *Upc2-1* strain. Our results suggest that the insertion of a transposon into the *TVP18* locus increased the level of *MOT3* mRNA, resulting in a transcriptionally mediated decrease in ABC transporter expression and a concom-

TABLE 3. Genes involved in transport-dependent accumulation of exogenous sterols

Cellular process	ORF	Gene	Function[a]	No. of isolates
Gene regulation and chromosome maintenance	YDR213w	*UPC2*	Transcription factor	14
	YKL160w	*ELF1*	Transcription elongation factor	1
	YMR106c	*YKU80*	Telomere length maintenance	1
	YDR448w	*ADA2*	Histone acetyltransferase subunit	1
Protein biosynthesis	RDN25-1	*RDN25*	Encodes rRNA	21
	YHR038w	*RRF1*	Ribosome recycling factor	1
Regulation of protein signaling	YMR032w	*HOF1*	Regulator of cytokinesis	1
	YNL098c	*RAS2*	GTPase, regulator of cell growth	2
	YDR436w	*PPZ2*	Phospho-Ser/Thr protein phosphatase	1
	YDL090c	*RAM1*	Farnesyltransferase subunit	1
	YER155c	*BEM2*	RhoGAP, cytoskeleton remodeling	1
	YLR361c	*DCR2*	Phosphatase, downregulation of UPR	1
Miscellaneous	YMR323w	*ERR3*	Predicted enolase	1
	YDL244w	*THI13*	Thiamine biosynthesis	1
	YCL017c	*NFS1*	Cysteine desulfurase, Fe/S cluster biosynthesis	1
Unknown function	YDR051c	*DET1*	Similarity to phosphoglycerate mutase	10
	YMR071c	*TVP18*	Membrane protein localized to late Golgi vesicles	10
	YPL158c	*AIM44*	Bud neck localized	1
	YFL040w		Putative transporter, member of the sugar porter family	1
	YKL027w		Localized to mitochondrial outer membrane	1
	YNR075w	*COS10*	DUP380 family member	1
	YGL083w	*SCY1*	Putative kinase	1
	YHR080c		May interact with ribosomes, copurifies with mitochondria	1

[a] Obtained from the *Saccharomyces* Genome Database (http://www.yeastgenome.org/).

itant decrease in sterol uptake. Indeed, analysis of the promoter regions of both *AUS1* and *PDR11* indicates numerous putative Mot3 binding sites and suggests that *AUS1* and *PDR11* can be included in the list of known anaerobic genes that are repressed by Mot3.

Expression and trafficking of Aus1 and Pdr11 are defective in some survivors. Since the suicide selection approach relies on efficient uptake of exogenous sterols, we anticipated that a number of the mutants that we recovered would have defects in the expression and trafficking of Aus1 and Pdr11 (40, 53). To examine this, Aus1-GFP and Pdr11-GFP fusion proteins (32) were expressed from their endogenous promoters in the mutants with transposon insertions in *HOF1*, *SCY1*, *RAM1*, *AIM44*, and *DET1*; *Upc2-1* cells expressing Aus1-GFP and Pdr11-GFP were used as a control. The localization and relative expression levels of the constructs were examined using fluorescence microscopy. Figure 4 shows that both Aus1-GFP and Pdr11-GFP were localized primarily to the PM in *Upc2-1* cells, consistent with their function in exogenous sterol uptake. However, the fluorescence of both Aus1-GFP and Pdr11-GFP was significantly reduced in all mutant strains examined except C209 (*det1*::mTn*3*), and in some strains, a large fraction of the

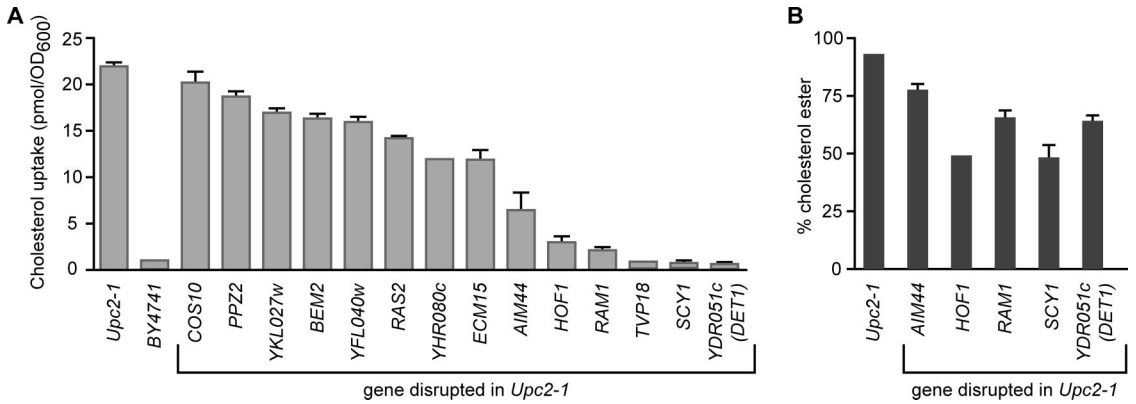

FIG. 2. Quantitative assay of sterol incorporation by a subset of suicide selection survivors. (A) Logarithmically growing cultures of isolates from the suicide selection were incubated in YPD with 1 μM cholesterol (containing 10 μCi [^{3}H]cholesterol) in 0.5% (wt/vol) ethanol–0.5% (wt/vol) Tween 80 for 60 min at 37°C. Cells were recovered, washed, and lysed. The incorporation of [^{3}H]cholesterol was determined by scintillation counting and extrapolated to the amount of bulk cholesterol uptake in the culture. The graph shows the averages and variances for two independent experiments. (B) Lysed cells from panel A were taken for lipid extraction; free cholesterol and cholesteryl esters in the extract were resolved by TLC and quantitated as described in Materials and Methods.

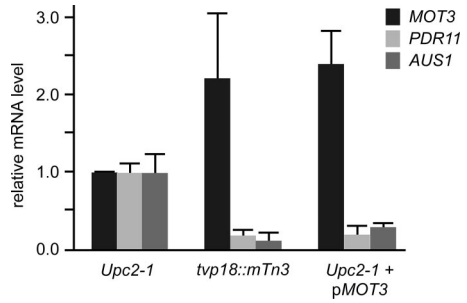

FIG. 3. Effect of Mot3 on expression of Aus1 and Pdr11. Logarithmically growing cultures of the *Upc2-1* strain, a mutant with a transposon insertion in the *TVP18* locus, and *Upc2-1* cells transformed with a single-copy plasmid containing *MOT3* (p*MOT3*) were preincubated at 37°C for 45 min. Cells were recovered, and RNAs were extracted and transcribed into DNA as described in Materials and Methods. After cDNA synthesis, the levels of mRNA corresponding to *MOT3*, *AUS1*, and *PDR11* were determined using QPCR. Data (normalized to results for the *Upc2-1* strain) show the averages and variances for two separate experiments.

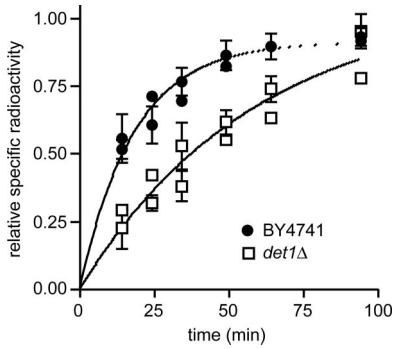

FIG. 5. Sterol transport in *det1*Δ cells. Sterol transport in BY4741 (parent) and *det1*Δ cells was measured by using MβCD to monitor the arrival of metabolically labeled ergosterol at the PM in a pulse-chase experiment as described in Materials and Methods. The RSR of [^{3}H]ergosterol in the MβCD extract was quantitated and graphed versus chase time. The lines represent mono-exponential fits of data averaged from at least two independent experiments. Error bars represent standard deviations.

fluorescence appeared to be localized to internal structures. Although it is not clear how these gene products regulate Aus1 and Pdr11 expression and trafficking, the decreased incorporation of exogenous sterols in strains carrying transposon insertions in *SCY1*, *AIM44*, *RAM1*, and *HOF1* is likely due to improperly sorted or expressed Aus1 and Pdr11 and not a result of a defect in sterol transport from the PM to the ER. In *det1::*mTn*3* cells, Aus1-GFP and Pdr11-GFP were detected in a pattern similar to that seen in *Upc2-1* cells. We conclude that the PM-localized sterol uptake machinery is normal in *det1::*mTn*3* cells. Together with the observation that sterol esterification is essentially unaffected when *DET1* is disrupted (Fig. 2B), this suggests that Det1 is likely to be involved in the transport of sterols from the PM to the ER.

ER-to-PM sterol transport is decreased in *det1*Δ cells. Since ergosterol transport between the ER and the PM occurs via a bidirectional process (3), we hypothesized that cells lacking *DET1* would be defective in biosynthetic sterol transport. To test this, we assayed transport of newly synthesized ergosterol from the ER to the PM in *det1*Δ cells obtained from the yeast nonessential gene deletion collection (54). Ergosterol was pulse labeled in the ER, using [^{3}H-methyl]methionine, and then chased with medium containing unlabeled methionine. Arrival of [^{3}H]ergosterol at the PM was measured by withdrawing an aliquot of cells and sampling PM sterol by extraction with MβCD. Ergosterol in both the MβCD extract and the corresponding cells was quantitated using reverse-phase HPLC. Radioactivity in the fractions corresponding to ergosterol was measured using scintillation counting to determine the SR in the sample. The RSR of ergosterol in the MβCD extract (the ratio of the SR in the MβCD extract to SR in the corresponding cell sample) was plotted against time to generate a transport curve (Fig. 5). ER-to-PM transport was significantly reduced in *det1*Δ cells: the initial rate of transport (determined from the linear portion [0 to 5 min] of a mono-exponential fit to the data) was reduced threefold in *det1*Δ cells relative to that in the parent strain BY4741. The cumulative data indicate that Det1 is involved, either directly or indirectly, in the bidirectional transport of sterols between the ER and the PM.

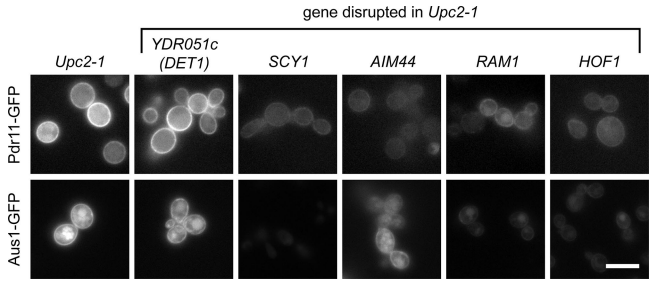

FIG. 4. Aus1-GFP and Pdr11-GFP localization. Mutants from the suicide selection were transformed with single-copy vectors bearing either *AUS1-GFP* or *PDR11-GFP* under the control of endogenous promoters. The strains were grown to mid-logarithmic phase in synthetic media lacking the appropriate nutrient and visualized using wide-field fluorescence microscopy. Images were generated under the same settings and processed identically. Representative cells are shown. Bar = 10 μm.

Preliminary characterization of cells lacking Det1. Compared to the parent WPY361 strain, the C209 *det1*::mTn*3* mutant did not display increased sensitivity or resistance to nystatin (5 to 50 U/ml on solid YPD), nor did it have any significant difference in sensitivity during growth with 0.5 M NaCl or 1.0 M KCl. However, C209 was about 10^4 times more temperature sensitive at 37°C and 10^2 times more cold resistant at 16°C than WPY361, as scored from serial dilutions spotted onto YPD plates (not shown). In liquid YPD medium, the C209 mutant had a doubling time of 99 min at 30°C, which is only slightly longer than that of the parent strain (88 min), while it grew slower at 37°C (doubling time of 138 min for the mutant and 96 min for the parent). Similarly, *det1*Δ cells grew slightly more slowly than the BY4741 parent strain but otherwise appeared normal, as judged by (i) transmission electron microscopy of negatively stained samples, (ii) calcofluor white staining (to detect cell wall defects), and (iii) uptake of the fluorescent lipophilic dye FM4-64 (to assess bulk endocytosis) (not shown). Since a sterol transport defect could lead to

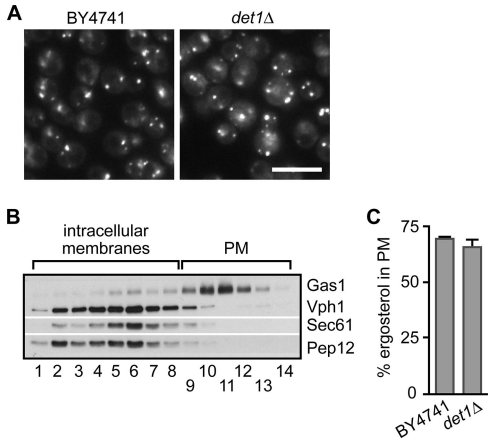

FIG. 6. Lipid droplets and subcellular distribution of ergosterol in *det1*Δ cells. (A) Lipid droplets in BY4741 (parent) and *det1*Δ cells were visualized by fluorescence microscopy after Nile red staining. Bar = 10 μm. (B) Fractionation of a BY4741 cell homogenate on a discontinuous RenoCal gradient as described in Materials and Methods. Fractions were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis immunoblotting, using antibodies against the PM (Gas1), vacuole (Vph1), ER (sec61), and Golgi complex/endosome (Pep12). Fractions 1 to 8, corresponding to intracellular membranes, and fractions 9 to 14, corresponding to the PM, were pooled and taken for lipid analysis. (C) Percentages of total ergosterol recovered in PM pools isolated from BY4741 and *det1*Δ cells.

changes in sterol content and intracellular distribution, we examined these properties of *det1*Δ cells (5, 11). Nile red staining showed a normal distribution of lipid droplets (Fig. 6A), with 2.6 ± 1.1 and 2.5 ± 1.2 lipid droplets per cell for *det1*Δ and BY4741 cells, respectively (mean ± standard deviation [SD]; >300 cells from each strain were examined). Similar results were obtained in a comparison of C209 and *Upc2-1* cells. Lipid analysis of cells grown to mid-logarithmic phase showed the same ergosterol/phospholipid molar ratio in *det1*Δ and BY4741 cells (0.187 ± 0.001 and 0.191 ± 0.019 for *det1*Δ and BY4741 cells, respectively [mean ± SD of duplicate measurements from two independent experiments]). We analyzed the subcellular distribution of ergosterol by using RenoCal gradients to separate the PM from other intracellular membranes in a cell homogenate. The quality of separation was assessed by immunoblotting with organelle-specific antibodies (Fig. 6B). Ergosterol in the separate pooled fractions was extracted with organic solvents and quantified using HPLC. The localization of ergosterol in *det1*Δ cells was nearly identical to that in the parent strain, with ~70% of the total sterol present at the PM (Fig. 6C).

DISCUSSION

Tritium suicide selection has been used successfully to isolate bacterial and yeast mutants with defects in lipid biosynthesis, protein N-glycosylation, and vesicular transport (8, 21, 22, 35, 37). We used this approach to identify genes involved in the uptake and intracellular transport of sterols. Both cholesterol and ergosterol are efficiently taken up by *Upc2-1* cells. Exogenously supplied ergosterol equilibrates with endogenous ergosterol in the PM and is consequently found mostly in the free (nonesterified) form in cells (32). Cholesterol partitions less well into the PM, possibly because it is excluded from privileged interactions with yeast sphingolipids (or other lipids with saturated acyl chains) when ergosterol is present. Consequently, exogenously supplied cholesterol transits through the PM and moves to the ER, where it is esterified by the acyl-coenzyme A cholesterol acyl transferase enzymes Are1 and Are2. We exploited this transport-dependent accumulation of exogenous cholesterol to perform [^{3}H]cholesterol suicide selection on *Upc2-1* cells mutagenized with a transposon library. We expected to recover mutants with defects in the uptake, transport, and esterification processes. From the survivors of the selection, 23 genes were identified; disruption of these genes by transposon insertion resulted in a defect in cholesterol uptake. Numerous genes were isolated only once (Table 3), indicating that coverage of the library was incomplete and that more mutants would likely be uncovered by extending or repeating the selection process. Of the genes we identified, *HOF1* and genes encoding components of the Ras signaling pathway (*RAS2* and *RAM1*) were also identified in a recent genomewide screen for genes involved in neutral lipid storage (11). Although the role of these gene products in sterol homeostasis is unclear, their repeated isolation not only validates both approaches—the published genomewide screen (11) and our suicide selection—for identifying new mutants but also confirms the importance of these proteins in intracellular sterol regulation. Interestingly, neither approach yielded mutants of *OSH3* or *OSH5*, which would be expected to display a reduced rate of cholesterol accumulation (39). Also, none of the genes that we identified were uncovered in a screen of anaerobic inviable mutants (40), even though this screen found numerous genes involved in the trafficking of Aus1 and Pdr11 to the PM. This lack of overlap is likely due to the incomplete coverage of the genome in our suicide selection process; by continuing the selection process, we would undoubtedly uncover a number of the reported anaerobic inviable mutants as well as mutants with transposon insertions in *OSH3* and *OSH5*.

Figure 1A envisages three steps in the accumulation of exogenously supplied cholesterol. C209 (*det1*::mTn*3*) cells esterify cholesterol similarly to parental *Upc2-1* cells and display a normal pattern of expression of Aus1 and Pdr11 (Fig. 4), suggesting that the defect in these cells is in the step of sterol transport between the ER and PM rather than in sterol uptake at the PM or sterol esterification. In support of this, assays of biosynthetic sterol transport in *det1*Δ cells revealed that transport of newly synthesized ergosterol from the ER to the PM was slowed threefold compared to that in parental BY4741 cells (Fig. 5).

Sequence analysis of the *det1::*mTn*3* mutant showed that transposon insertion occurred between chromosomal coordinates 557748 and 557749 on chromosome IV, allowing for the production of a truncated protein corresponding to the first 104 amino acids of Det1. Preliminary results indicated that this truncated protein acts in a dominant-negative way to inhibit sterol uptake (not shown), potentially accounting for the severity of the defect (~20-fold) in the rate of sterol accumulation in C209 cells (Fig. 2) compared with the milder defect (~3-fold reduction) in the rate of biosynthetic sterol transport in *det1*Δ cells (Fig. 5).

How might Det1 play a role in nonvesicular sterol transport? Det1 is an ~39-kDa soluble protein with a predicted abun-

dance of ~3,000 molecules per cell (20). GFP-tagged Det1 is localized primarily to the cytoplasm (12, 23), consistent with a role in sterol transport across the cytoplasm. Sequence analysis shows that Det1 has significant similarity to phosphoglycerate mutases. However, Det1 lacks a number of residues shown to be critical for catalysis (51, 52), and this activity (as performed by Gpm1, Gpm2, and/or Gpm3) in *S. cerevisiae* has been well characterized (17, 41). Other than the predicted similarity to phosphoglycerate mutases, sequence analysis reveals that Det1 has a number of close homologs in other fungi (i.e., KLLA0F18810g in *Kluyveromyces lactis* and ca5949 in *Candida albicans*) but no clear homologs in mammals. Perhaps Det1 is involved in a yeast-specific or ergosterol-specific aspect of sterol transport or significant sequence divergence has occurred in the course of evolution of the more complex cholesterol regulatory mechanisms in higher eukaryotes. While the identification of Det1 as an important player in intracellular sterol transport opens the door to future studies on this poorly understood process, more analyses are required in order to determine the exact nature and mechanism of its involvement.

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