Supporting Information

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Methods

Strains. Gene replacements and GFP C-terminal fusions were generated with the PCR transformation technique (1) and confirmed by PCR. Plasmid pJV3, a pRS416 derivative bearing SNC1-GFP under the TPI1 promoter, was a kind gift from Javier Valdez-Taubas (Universidad Nacional de Cordoba, Argentina). *ERG4* was cloned in EagI and BamHI sites pRS316 by using the following oligos: 5′-aattatcggccgtgcaaattgtctttttttagc-3′ and 5′-aattggatccgtatggacacgtttcatttagg-3′. *ERG5* was cloned in EagI and BamHI sites pRS315 by using the following oligos: 5′-aattacggccgaacacttctttcctttg-3′ and 5′-atttaggatcctgcaaaaagattagctg-3′.

Genetic Screen for Enhancers of *prm1*Δ. Four A_{600} units of *prm1*Δ *TRP1 MAT***a** cells were incubated with the mutagen ethylmethanesulfonate (Sigma-Aldrich) for 30 min at 30 °C. At that point, the reaction was quenched by 10% sodium thiosulfate (Sigma-Aldrich) addition. Cells were washed twice in YPD medium and allowed to recover in YPD for 90 min at 30 °C. Serial dilutions of this stock were plated to medium lacking tryptophan, and the titer of colony-forming units was calculated; meanwhile, the stock was kept at 4 °C.

For screening, the stock was plated to 100 plates lacking tryptophan at a density of 120 colonies per plate. Colonies were allowed to grow for ∼40 h at 30 °C. After ∼25 h, a stationary overnight culture of the *prm1*Δ *kex2*Δ *URA3 MAT*α strain was plated to 100 plates of YPD at 100 μL per plate and was incubated at room temperature for the remaining 15 h to form lawns. These lawns were respread with 100 μL per plate of water to a dull matte appearance indicative of homogeneity. Colonies of the mutagenized *MAT***a** cells were replica-plated to mating lawns and incubated for 8 h at 30°C. The plates were then replica-plated to medium lacking tryptophan and uracil to select for diploids. Phenotypes were scored on plates incubated for 2 days at 30 °C. After backcross to a Δ*prm1* strain, the strongest Δ*prm1 MAT***a** mutant (A3) was transformed with a pRS316-based library and ∼15,000 transformants were subjected to a replica mating assay as described in ref. 2. Plasmids from suppressed clones were isolated and retested on the A3 mutant before sequencing.

Quantitative Cell Fusion and Shmooing Assays. Cells of opposite mating types, in which one expresses soluble cytosolic green fluorescent protein [GFP, plasmid pDN291 (3)], were grown to mid-logarithmic growth phase. An equal number of cells of each mating type were mixed and vacuumed to a nitrocellulose filter. The filter was placed cell-side-up on YPD plates and then incubated for 3 h at 30 °C. Cells were scraped off of the filter, fixed in 4% paraformaldehyde, incubated at 4 °C overnight, and inspected by fluorescence microscopy. For each mating, four independent experiments were performed, and at least 100 mating pairs were scored.

β-Galactosidase Assays. Yeast strains containing the *FUS1-lacZ* reporter were grown to mid-logarithmic growth phase in YPD and incubated with 10 μg/mL α-factor for the indicated times. Reporter activity was quantified by using the Yeast β-Galactosidase Assay Kit (Pierce) as described in ref. 2.

Microscopy. Wide-field fluorescence and differential interference contrast (DIC) microscopy were performed by using an Axiovert 200M microscope (Zeiss), equipped with an X-cite 120 mercury arc lamp (EXFO) and an Orca ER camera (Hamamatsu). Image-Pro (Media Cybernetics) was used for data collection. Confocal fluorescence microscopy was performed with a Zeiss LSM510 apparatus. Live cell microscopy was performed by mounting yeast cells on 1 mg/mL concanavalin-A (Sigma)-treated coverslips in complete synthetic medium.

Filipin Staining. Live cells were incubated in complete synthetic medium with 9 μg/mL filipin (in DMSO) for 15 min at room temperature. Cells were washed three times in medium and mounted for confocal microscopy in the same medium.

Sterol Purification and Mass Spectrometry. Yeast total lipids were extracted by the Bligh & Dyer protocol (4). Briefly, 100 ODs of yeast cells were collected and resuspended in 3 mL of 3 mM NaN_3. Then, 20 mL of $CHCl_3$/MetOH (1/1) were added and the mixture was vortexed for 3 min. After centrifugation for 3 min at 3,000 rpm (1,310 × *g*), the supernatant fraction was saved and lipids were re-extracted with 10 mL of $CHCl_3$/MetOH/H_2O (10/10/3). The resulting organic phase was dried under N_2 gas and stored at −20 °C.

For free sterol purification, silica chromatography was performed by using 24:1 $CHCl_3$:MetOH as the running solvent. Sterols were followed by analytic TLC, using 2.5% (wt/vol) ceric ammonium sulfate stain.

For mass spectrometry analysis, sterol fractions were diluted 40-fold in $CHCl_3$/MeOH/2-propanol 1/2/4 (vol/vol/vol) containing 5 mM ammonium acetate. Before the analysis, samples were vortexed thoroughly and centrifuged for 5 min at 14,000 rpm on a Minispin centrifuge (Eppendorf). All measurements were performed on a modified QSTAR Pulsar i quadrupole time-of-flight mass spectrometer (MDS Sciex) equipped with an automated nanospray chip ion source NanoMate HD (Advion BioSciences).

Acquired spectra were interpreted with Analyst QS 1.1 (MDS Sciex). Sterol species were identified by accurate mass measurements and MS/MS analysis. Theoretical and experimental determined *m*/*z* values for the [M+H], [M+NH_4], and [M+H-H_2O] ions are displayed in Table S1. MS/MS experiments with low collision energy were performed on [M+NH_4] precursor ions for detection of the characteristic [M+H-H_2O] fragment ions.

For semiquantitative interpretation, peak areas of the [M+H] of the identified sterol species were extracted, and afterward a correction for overlapping isotopic peaks was performed manually (Figs. S2 and S3). The resulting corrected values were normalized according to the sum of all detected sterols.

1. Longtine MS, et al. (1998) Additional modules for versatile and economical PCR-based gene deletion and modification in Saccharomyces cerevisiae. *Yeast* 14:953–961.
2. Heiman MG, Engel A, Walter P (2007) The Golgi-resident protease Kex2 acts in conjunction with Prm1 to facilitate cell fusion during yeast mating. *J Cell Biol* 176: 209–222.
3. Heiman MG, Walter P (2000) Prm1p, a pheromone-regulated multispanning membrane protein, facilitates plasma membrane fusion during yeast mating. *J Cell Biol* 151: 719–730.
4. Bligh EG, Dyer WJ (1959) A rapid method of total lipid extraction and purification. *Can J Biochem Physiol* 37:911–917.

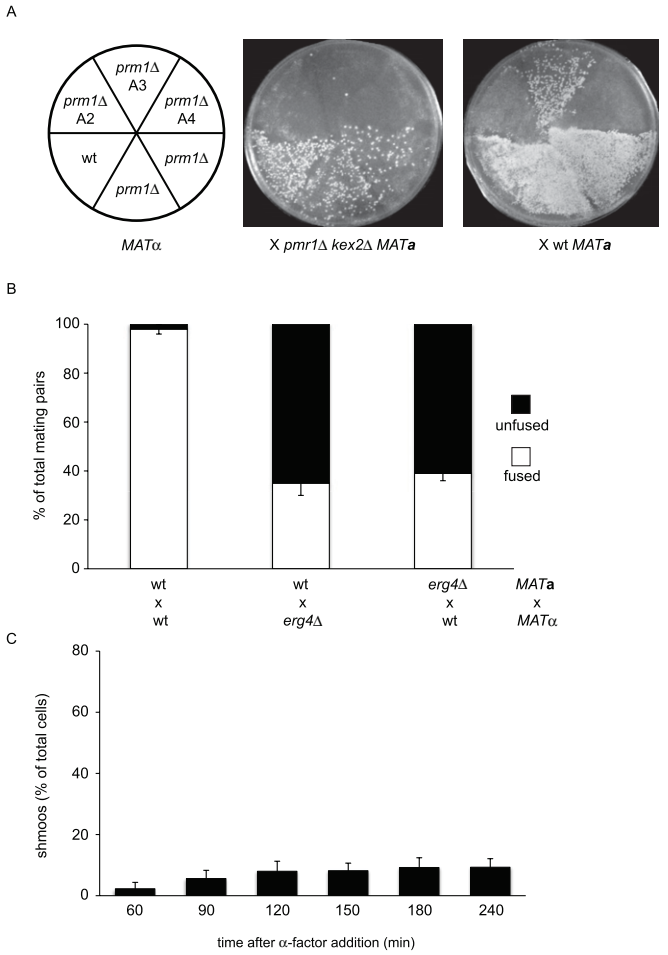

Fig. S1. Characterization of the A3/*erg4* mutant. (*A*) Candidate mutants and control *MAT*α strains were streaked on YPD plates, grown overnight at 30 °C, and replica-plated to lawns of *prm1*Δ *kex2*Δ and wild-type *MAT***a** strains. After mating, mixtures were replica-plated to selective media for diploid selection. Those candidates that did not yield diploids when mated against the wild-type strain were discarded as sterile mutants (i.e., A2 and A4). Resulting diploids are shown. (*B*) Quantitative cell fusion assays with wild-type, *erg4::HIS3 MAT***a**, and *MAT*α strains were performed as described in *SI Methods*. Error bars indicate SEs. (*C*) Shmoo formation efficiency of *erg4::HIS3 MAT***a** strain was performed as described in *SI Methods*.

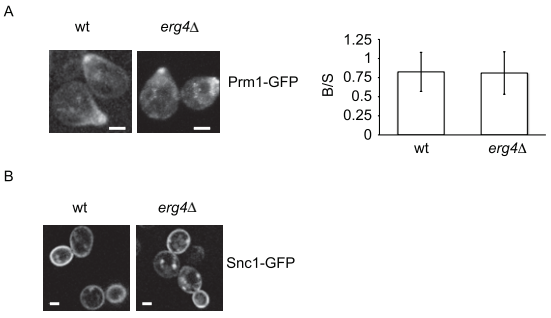

Fig. S2. (*A*) *erg4*Δ cells show normal polarization of Prm1-GFP. Wild-type and *erg4*Δ *MAT***a** strains harboring Prm1-GFP were grown on YPD and then treated with α-factor for 3 h, fixed, and imaged by confocal microscopy. Fluorescence distribution along the longitudinal axis of cells was quantified as the fluorescence intensity ratio between the shmoo (S) and body (B) areas as described in *Materials and Methods*. Error bars indicate SEs. Representative sum *z*-projections are shown. (Scale bars: 2 μm.) (*B*) *erg4*Δ cells show normal polarization of Snc1. Wild-type and *erg4*Δ *MAT***a** strains harboring Snc1-GFP were grown on YPD and then fixed before imaging. (Scale bars: 2 μm.)

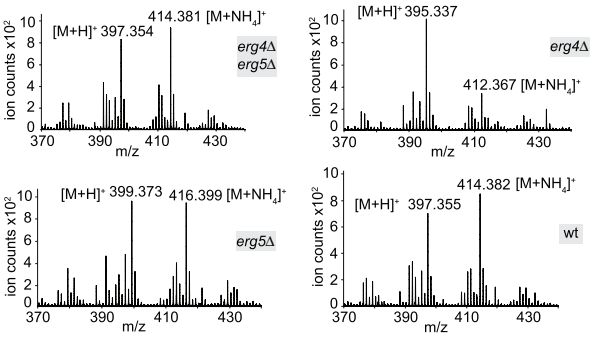

Fig. S3. Mass spectra of the different accumulated sterols (370–440 *m/z* range is shown). Yeast cells from the different strains were harvested, and total lipids were extracted. Free sterols were further purified and analyzed by MS/MS. Peaks of the predominant protonated molecular as well as ammonia adduct species are indicated.

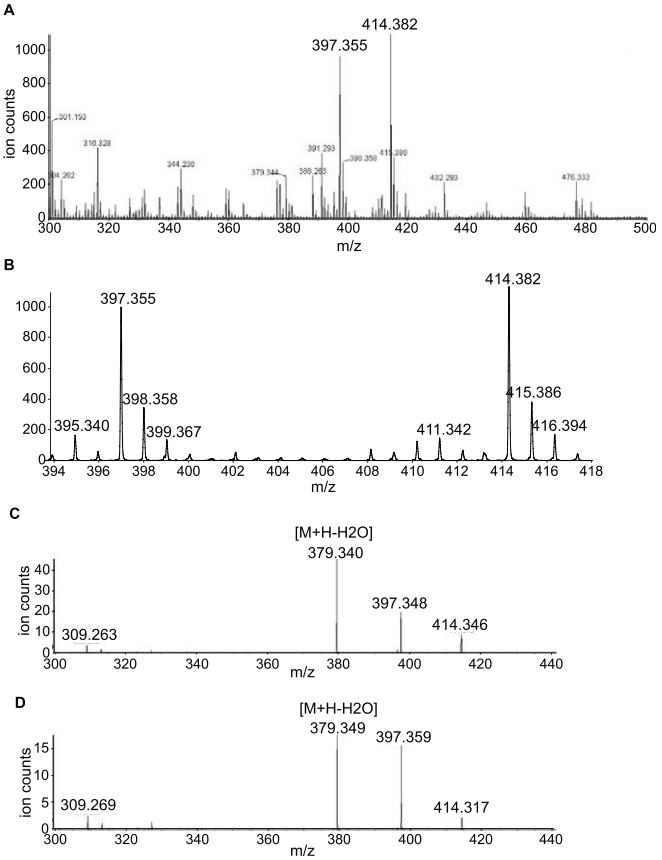

Fig. S4. Representative spectra of TLC purified free sterol fractions. (*A*) MS1 of wild-type strain showing within the chemical background the protonated ergosterol at *m/z* 397.355 and the ammonia adduct at *m/z* 414.382. (*B*) Zoom in to the *m/z* range 394–418. The spectra are overlaid with the theoretical isotopic distributions for the protonated ergosterol and its ammonia adduct. (*C*) MS/MS spectra of the ammonia adduct from ergosterol standard and from the wild type (*D*). The characteristic [M+H-H_2O] ion is indicated. Theoretical and experimental data are further presented in Table S1.

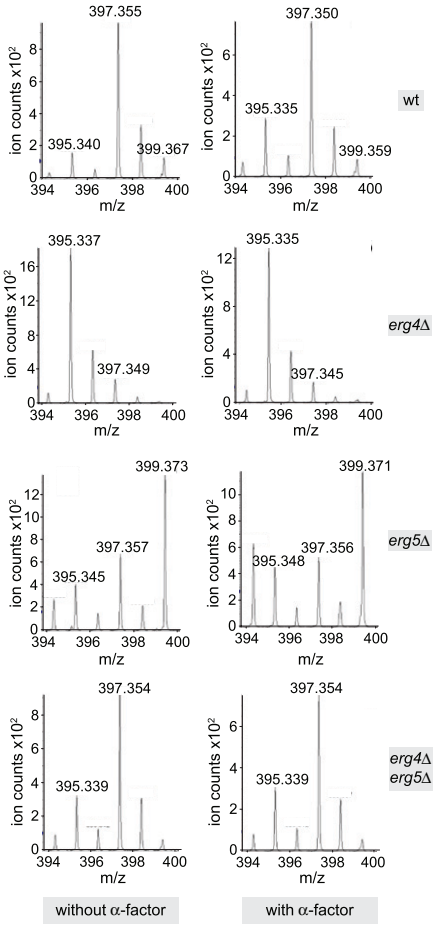

Fig. S5. Enlargement of the *m/z* region (394–400) of protonated sterols used for semiquantitative analysis of sterol composition of different strains incubated in the absence (*Left*) or presence (*Right*) of α-factor.

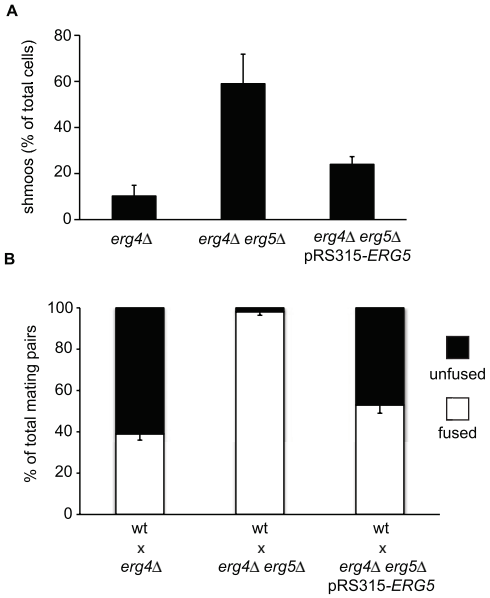

Fig. S6. *ERG5* complementation of the *erg5*Δ *erg4*Δ strain. (*A*) Cells growing on YPD were treated with α-factor for 3 h, fixed, and examined under the microscope for quantification of shmooing efficiency. Error bars indicate SEs. (*B*) Quantitative cell fusion assays were performed as already described. Error bars indicate SEs.

Table S1. Strains used in this study

Strain	Genotype	Source
W303-1A	*MAT***a** *leu2-3,112 trp1-1 can1-100 ura3-1 ade2-1 his3-11,15*	Walter lab
W303-1B	*MAT*α *leu2-3,112 trp1-1 can1-100 ura3-1 ade2-1 his3-11,15*	Walter lab
PSAY74	*MAT***a**, pDN291	Walter lab
PSAY75	*MAT*α, pDN291	Walter lab
MHY426	*MAT***a** *prm1*Δ*::HIS3*	Walter lab
MHY427	*MAT***a** *prm1*Δ*::HIS3 kex2::TRP1*	Walter lab
MHY191	*MAT*α *prm1* Δ*::HIS3*, pDN291	Walter lab
PSAY144	*MAT***a** *erg4*Δ*::HIS3*	This study
PSAY179	*MAT*α *erg4*Δ*::HIS3*, pDN291	This study
PSAY87	*MAT***a** *prm1*Δ*::HIS3 erg4*Δ*::KanMX4*	This study
PSAY95	*MAT*α *prm1*Δ*::HIS3 erg4*Δ*::KanMX4*, pDN291	This study
PSAY107	*MAT***a**, p*FUS1-LacZ::URA3*	This study
PSAY108	*MAT***a** *erg4*Δ*::KanMX4*, p*FUS1-LacZ::URA3*	This study
PSAY546	*MAT***a**, *PGK1-mCherry::HIS3*	This study
PSAY450	*MAT***a** pRS316-*FIG1-GFP*	This study
PSAY451	*MAT***a** *erg4*Δ*::HIS3*, pRS316-*FIG1-GFP*	This study
MHY153	*MAT***a**, *PRM1-GFP::HIS3*	This study
PSAY93	*MAT***a**, *erg4*Δ*::KanMX4 PRM1-GFP::HIS3*	This study
PSAY497	*MAT***a**, *SHS1-GFP::HIS3*	This study
PSAY499	*MAT***a**, *erg4D::cgLEU2* SHS1-GFP::HIS3*	This study
PSAY833	*MAT***a**, pJV3	This study
PSAY834	*MAT***a**, *erg4D::cgLEU2*,* pJV3	This study
PSAY180	*MAT***a**, *erg5::KanMX4*	This study
PSAY181	*MAT*α, *erg5::KanMX4*, pDN291	This study
PSAY176	*MAT***a**, *erg4*Δ*::HIS3 erg5::KanMX4*	This study
PSAY183	*MAT*α, *erg4*Δ*::HIS3 erg5::KanMX4*, pDN291	This study
PSAY222	*MAT***a**, *erg4*Δ*::HIS3 erg5::KanMX4*, pRS316-*ERG5*	This study

**cgLEU2* indicates *Candida glabrata LEU2* gene.

Table S2. Identification of sterols by direct infusion experiments by accurate mass measurements

	$[M+H]^+$		$[M+NH4]^+$		$[M+H - H_2O]^+$*	
	Theoret.	Exp.	Theoret.	Exp.	Theoret.	Exp.
Ergosta-5,7,-dienol	399.362	399.372	416.389	416.398	381.352	381.362
Ergosta-5,7,24(28)-trienol	397.346	397.354	414.373	414.381	379.336	379.344
Ergosterol	397.346	397.353	414.373	414.379	379.336	379.345
Ergosta-5,7,22,24(28)-tetraenol	395.331	395.336	412.357	412.366	377.32	377.325

*Determined from MS/MS of the sterol ammonia adduct [M+NH4].