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New Regulators of a High Affinity Ca^{2+} Influx System Revealed through a Genome-wide Screen in Yeast*[S]

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The bakers' yeast *Saccharomyces cerevisiae* utilizes a high affinity Ca^{2+} influx system (HACS) to survive assaults by mating pheromones, tunicamycin, and azole-class antifungal agents. HACS consists of two known subunits, Cch1 and Mid1, that are homologous and analogous to the catalytic α-subunits and regulatory $\alpha 2\delta$-subunits of mammalian voltage-gated calcium channels, respectively. To search for additional subunits and regulators of HACS, a collection of gene knock-out mutants was screened for abnormal uptake of Ca^{2+} after exposure to mating pheromone or to tunicamycin. The screen revealed that Ecm7 is required for HACS function in most conditions. Cycloheximide chase experiments showed that Ecm7 was stabilized by Mid1, and Mid1 was stabilized by Cch1 in non-signaling conditions, suggesting they all interact. Ecm7 is a member of the PMP-22/EMP/MP20/Claudin superfamily of transmembrane proteins that includes γ-subunits of voltage-gated calcium channels. Eleven additional members of this superfamily were identified in yeast, but none was required for HACS activity in response to the stimuli. Remarkably, many dozens of genes involved in vesicle-mediated trafficking and protein secretion were required to prevent spontaneous activation of HACS. Taken together, the findings suggest that HACS and calcineurin monitor performance of the membrane trafficking system in yeasts and coordinate compensatory processes. Conservation of this quality control system in *Candida glabrata* suggests that many pathogenic species of fungi may utilize HACS and calcineurin to resist azoles and other compounds that target membrane biosynthesis.

Nearly all fungi contain a gene whose protein product is homologous to the catalytic α-subunits of voltage-gated Ca^{2+} channels (VGCCs)[2] in animals (1, 2). VGCCs typically sense transmembrane electrical potentials and pass Ca^{2+} in response to electrical depolarization (for review, see Ref. 3). The fungal homologs, such as Cch1 in *Saccharomyces cerevisiae*, contain half as many cationic residues in their voltage-sensing S4 segments as mammalian homologs, suggesting channel gating and Ca^{2+} influx in fungi may respond to an unusual voltage range or to other kinds of stimuli.

In *S. cerevisiae*, Cch1 functions as part of a high affinity Ca^{2+} influx system (HACS) that becomes activated very rapidly in response to sudden increases in the pH of the environment (4). A much slower HACS activation is observed during prolonged exposure to mating pheromones (5–7) or to compounds such as azole-class antifungal agents that inhibit essential enzymes in the endoplasmic reticulum (8–12). In all these instances, Cch1 is important or required for activation of calcineurin, which is necessary for appropriate adaptation and survival of the cells in these conditions (for review, see Ref. 13). Thus, HACS and calcineurin constitute an important defense mechanism used by fungi to resist certain kinds of toxins and some common antifungal agents.

The subunit composition of HACS and the various mechanisms that regulate this important Ca^{2+} transporter are not well understood. In *S. cerevisiae* and other yeasts, HACS also requires Mid1, a protein with sequence homologs only in fungi and a secondary structural that is similar to $\alpha 2\delta$-subunits of animal VGCCs. Cch1 and Mid1 form a detergent-stable complex (8), and both proteins are necessary for Ca^{2+} influx, elevation of cytosolic free Ca^{2+} ($[Ca^{2+}]_{cyt}$), and activation of calcineurin in response to most stimuli. Interestingly, calcineurin appears to dephosphorylate the Cch1 subunit of HACS and to feedback-inhibit HACS activity in response to slow-acting stimuli such as tunicamycin, miconazole, and the mating pheromone α-factor (6, 8). The MAP kinase Slt2 was required for HACS activation in response to tunicamycin (14), and the MAP kinase Fus3 was required for HACS activation in response to mating pheromone (15). The critical sites of phosphorylation and the molecular mechanism of HACS regulation by kinases and phosphatases are not yet known.

To improve our understanding of HACS composition and regulation, we screened a collection of yeast mutants lacking non-essential genes for defects in Ca^{2+} uptake during responses to mating pheromones and tunicamycin in both the presence and absence of a calcineurin inhibitor (FK506). The screen yielded the uncharacterized protein Ecm7 as a probable new subunit of HACS. Ecm7 was found to be homologous to γ-subunits of VGCCs and other members of the PMP-22/EMP/MP20/Claudin superfamily of proteins, which number at least 40 in humans (16) and at least 12 in *S. cerevisiae*. The screen also revealed many mutants that exhibit spontaneous HACS

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[S] The on-line version of this article (available at http://www.jbc.org) contains supplemental Table 1.

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[2] The abbreviations used are: VGCC, voltage-gated Ca^{2+} channel; LACS, low affinity Ca^{2+} influx system; HACS, high affinity Ca^{2+} influx system; YPD, yeast extract/peptone/dextrose.

TABLE 1
Yeast strains used in this study

Name[a]	Genotype	Strain[a]	Source
K601		W303-1A	(7)
ELY117	*cch1*::*TRP1*	W303-1A	(7)
ELY138	*mid1*::*LEU2*	W303-1A	(7)
LMY005	*ecm7*::*NatR*	W303-1A	
ELY151	*cch1*::*TRP1 mid1*::*LEU2*	W303-1A	(7)
VBY205	*cch1*::*TRP1 ecm7*::*NatR*	W303-1A	
VBY202	*mid1*::*LEU2 ecm7*::*NatR*	W303-1A	
K1619	*ECM7-MYC*::*TRP1*	W303-1A	
K1621	*ECM7-MYC*::*TRP1 mid1*::*LEU2*	W303-1A	
K1623	*ECM7-MYC*::*TRP1 cch1*::*HIS3*	W303-1A	
K1625	*ECM7-MYC*::*TRP1 cch1*::*HIS3 mid1*::*LEU2*	W303-1A	
K1643	*CCH1-MYC*::*TRP1*	W303-1A	
K1645	*CCH1-MYC*::*TRP1 mid1*::*LEU2*	W303-1A	
K1647	*CCH1-MYC*::*TRP1 ecm7*::*NatR*	W303-1A	
K1649	*CCH1-MYC*::*TRP1 mid1*::*LEU2 ecm7*::*NatR*	W303-1A	
K1659	*MID1-MYC*::*G418R*	W303-1A	
K1661	*MID1-MYC*::*G418R cch1*::*TRP1*	W303-1A	
K1663	*MID1-MYC*::*G418R ecm7*::*NatR*	W303-1A	
K1665	*MID1-MYC*::*G418R cch1*::*TRP1 ecm7*::*NatR*	W303-1A	
YSE700		BY4741	(18)
YSE701		BY4742	(18)
YSE789	*cch1*::*G418R*	BY4741	(18)
YSE788	*cch1*::*G418R*	BY4742	(18)
YSE481	*mid1*::*G418R*	BY4741	(18)
YSE482	*mid1*::*G418R*	BY4742	(18)
YSE796	*ecm7*::*G418R*	BY4741	(18)
YSE795	*ecm7*::*G418R*	BY4742	(18)
YSE702	*fig1*::*G418R*	BY4741	(18)
YSE703	*fig1*::*G418R*	BY4742	(18)
YSE791	*fig1*::*G418R cch1*::*G418R*	BY4741	
YSE792	*fig1*::*G418R cch1*::*G418R*	BY4742	
YSE756	*fig1*::*G418R mid1*::*G418R*	BY4741	
YSE757	*fig1*::*G418R mid1*::*G418R*	BY4742	
YSE797	*fig1*::*G418R ecm7*::*G418R*	BY4741	
YSE798	*fig1*::*G418R ecm7*::*G418R*	BY4742	
BG2		BG2	(9)
Cgcch1	*cch1*::*URA3(Tn7)*	BG2	(9)
Cgmid1	*mid1*::*URA3(Tn7)*	BG2	(9)
Cgecm7	*ecm7*::*URA3(Tn7)*	BG2	(9)

[a] W303-1A (*S. cerevisiae MAT***a** *ade2-1 can1-100 his3-11,14 leu2-3,112 trp1-1 ura3-1*), BY4741 (*S. cerevisiae MAT***a** *his3Δ1 leu2Δ0 met15Δ0 ura3Δ0*), BY4742 (*S. cerevisiae MATα his3Δ1 leu2Δ0 lys2Δ0 ura3Δ0*), BG2 (*C. glabrata ura3Δ::Tn903NeoR*).

activation in the absence of external stimuli. Many of these mutants have defects that perturb membrane trafficking and secretory protein biogenesis, which is rather similar to the effects of tunicamycin and azole-class antifungal agents. Many of these mutants also require HACS and calcineurin for viability. This study adds insights into the structural composition and regulation of HACS/VGCC-related Ca^{2+} channels and also reveals a critical role for HACS and calcineurin in quality control of secretory and membrane trafficking systems of *S. cerevisiae* and perhaps other fungi.

EXPERIMENTAL PROCEDURES

Yeast Strains, Culture Media, and Reagents—The *S. cerevisiae* and *Candida glabrata* strains used in this study (Table 1) were obtained from original sources or derived from parental strain W303-1A (17), BY4741/BY4742 (18), or BG2 (9) using standard genetic crosses or PCR-based methods for introduction of knock-out mutations and epitope tags (19). Yeast strains were cultured in rich YPD medium or synthetic SC medium (20). Purified synthetic α-factor mating pheromone was obtained from the Johns Hopkins University Synthesis and Sequencing Facility and dissolved in DMSO. Tunicamycin was purchased from Sigma and dissolved in methanol or DMSO. FK506 was obtained from Astellas Pharma and dissolved in DMSO. Aqueous $^{45}CaCl_2$ was purchased from MP Biosciences.

Genome-wide Screen for Mutants with Altered Uptake of Ca^{2+}—A collection of 4848 different gene knock-out mutants of *S. cerevisiae* strain BY4741 (genotype *MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0*) (18) was obtained from Invitrogen and stored at −80 °C. Each of these strains was thawed, pinned into flat-bottom 96-well dishes containing 180 μl of YPD culture medium, and grown for 2 days at room temperature. After resuspending the stationary phase cultures using a vortex mixer, six 15-μl aliquots were transferred using a 12-channel micropipette to six filter-bottom 96-well dishes (Multiscreen HTS HV; Millipore) containing 100 μl of fresh YPD medium with trace quantities of $^{45}CaCl_2$ (~4 μCi/ml final) and either α-factor mating pheromone (40 μM final), tunicamycin (5 μg/ml final), or solvent controls (2.5% DMSO, 0.5% methanol) in both the absence and presence of FK506 (1 μg/ml final). The cultures were mixed and incubated for 6 h at room temperature, and the cells were harvested by vacuum filtration, washed 5 times with 150 μl buffer A (5 mM Na-HEPES, pH 5.5, 10 mM $CaCl_2$), dried overnight at room temperature, and analyzed in a TopCount-NXT liquid scintillation counter (Packard Instrument Co.) after adding 100 μl of Opti-Fluor scintillation mixture (PerkinElmer Life Sciences). Twelve subsets of the knock-out collection (with partial overlap) were assayed in this manner on 12 different days. To correct for batch-to-batch variation, the robust multivariate center of each batch was determined (21) using the R statistical package. Each batch was then multiplied by a scaling factor that translates the robust means into a reference mean, which was arbitrarily set at 1 for the untreated cultures. This procedure removed all systematic batch-to-batch variation and merged the entire dataset, which is presented in supplemental Table 1.

The normalized data were filtered to remove mutants with wild-type phenotypes. The non-wild-type mutants were then organized into phenotypic groups by hierarchical clustering of their Ca^{2+} uptake profiles. Briefly, each mutant strain was projected as a single point in six-dimensional space, and each point exhibiting a Mahalanobis distance (22) from the center greater than 36.75 units (corresponding to the χ^2 value with a significance level) was considered to be significantly different from wild type. This first filter eliminated 4152 of the 4955 points. A second filter was designed to identify cultures that had atypically low or high cell density during the assay but otherwise displayed wild-type patterns of Ca^{2+} uptake in the six conditions tested. Briefly, the angle between the origin and each point in six-dimensional space was calculated, and all points less than 3 S.D. from the average included angle were identified. This second filter eliminated an additional 44 points, leaving a total of 759 mutant strains that produced non-wild-type Ca^{2+} uptake profiles. It should be noted that 13 strains with apparently low cell density escaped this second filter and were included in subsequent analyses.

Certain mutant strains are known to exhibit high Ca^{2+} uptake in the absence of exogenous α-factor or tunicamycin stimulation, a fact that complicates the analysis of stimulus-induced Ca^{2+} uptake. To identify such mutants, we calculated Mahalanobis distances as before but using only two (of six) experimental conditions lacking α-factor and tunicamycin and found that 187 of the 759 remaining mutant strains exceeded

the 95% significance level (Mahalanobis distance greater than 26 units). These mutants (Group A) exhibited altered Ca^{2+} uptake in YPD medium or YPD plus FK506 medium, whereas the remaining 572 mutants (Group B) exhibited altered Ca^{2+} uptake profiles in the presence of α-factor or tunicamycin. The mutants in Group A and Group B were separately organized by hierarchical clustering (Matlab) using Pearson correlation coefficients. The data in supplemental Table 1 were sorted according to the cluster position and were analyzed further as described under "Results."

Quantitative Ca^{2+} Uptake Experiments—Total cellular Ca^{2+} accumulation was measured as described previously (23). Briefly, cells were grown to log phase in YPD medium overnight, harvested, and resuspended in fresh YPD medium supplemented with trace quantities (~10 μCi/ml) of $^{45}CaCl_2$ and various compounds. After a 2-h incubation in a 96-well filtration plate (Millipore) at 30 °C, cells were harvested and washed 3 times with ice-cold buffer A (10 mM $CaCl_2$, 5 mM Na-HEPES, pH 6.5) using a vacuum filtration unit (Millipore). The filters were dried at room temperature, covered with Microscint20 scintillation mixture (PerkinElmer Life Sciences), and counted using a TopCount NXT (Packard Instrument Co.) scintillation counter.

Aequorin Luminescence Experiments—The appropriate yeast strains (K601, ELY117, ELY138, and LMY005) were transformed with the aequorin expression plasmid (pKC147) (7), grown to log phase, adjusted for cell density, loaded with coelenterazine (Molecular Probes, Inc., Eugene, OR), and suspended in fresh YPD medium as described previously (7). After 30 s of incubation at room temperature in a tube luminometer (LB9507, EG&G Wallac), an equal volume of YPD medium adjusted to pH 9.0 with sodium hydroxide was injected into each culture. Luminescence was recorded at 0.2-s intervals for an additional 2.5 min. The raw data were normalized to a common cell density.

Western Blot Experiments—The appropriate yeast strains were cultured at 30 °C in YPD medium to log phase, adjusted to an OD_{600} of 0.5, and split into two aliquots. One aliquot was treated with 10 μg/ml cycloheximide (Sigma) for 1 h at 30 °C before processing, and the other was processed immediately. Processing involved centrifugation of 2.5 A_{600} units of cells at 4 °C, lysis of cells in the presence of protease inhibitors using glass beads, extraction of proteins with urea sample buffer, SDS-PAGE, and Western blotting as described previously (8).

Identification of Ecm7 Homologs—Supervised BLAST and PSI-BLAST searches of current databases were performed using default settings (24). This approach revealed seven homologs of Ecm7 in *S. cerevisiae* that are all listed as members of the Sur7/PalI superfamily (pfam06687). Three additional members of the superfamily (Rim9/YMR063w, YFR012w, YOL019w) were not found, even after 10 iterations. PSI-BLAST searches using Rim9 and Sur7 as queries yielded seven and eleven members of this superfamily, respectively. To search for extremely divergent members of this superfamily that might have been missed by PSI-BLAST, the 11 known members of the superfamily were parameterized using 6 different features of their secondary structures, and the median of this set was used for comparison against a similarly parameterized proteome of *S. cerevisiae*. The six quantitative parameters were the number of amino acids between (*a*) the N terminus and the first predicted transmembrane segment, (*b*) the first and second transmembrane segments, (*c*) the second and third transmembrane segments, (*d*) the third and fourth transmembrane segments, (*e*) the ultimate and penultimate cysteine residues in the first extracellular loop, and (*f*) the ultimate cysteine residue in the first extracellular loop and the beginning of the second transmembrane segment. A list of transmembrane proteins in *S. cerevisiae* (available upon request) was generated using a previously described method (25) and kindly provided to us by Dr. John Ward. Out of 1333 predicted transmembrane proteins in the *S. cerevisiae* proteome, only 12 were found to contain four predicted transmembrane segments and a Pearson correlation coefficient greater than 0.95 to the median parameter set. The only new member, Sma2 (correlation coefficient >0.99) and its homologs in other yeasts exhibited a pattern of sequence conservation similar to the other members of the Sur7/PalI superfamily (data not shown), which suggests that Sma2 is the 12th member of the Sur7/PalI and PMP-22/EMP/MP20/Claudin superfamilies in *S. cerevisiae*.

Mating Assays—Lawns of *MATα* strains and patches of *MAT***a** strains were grown on low calcium synthetic complete medium containing Noble agar. *MAT***a** strain patches and *MATα* lawns were replica-plated together onto the same medium and allowed to mate for 4 h at 30 °C. The cells were then replica-plated to the same medium lacking methionine and lysine to select for mated cells (diploids). Plates were photographed after 1 day of growth. Similar results were obtained when matings were performed in medium containing 20 mM $CaCl_2$ (data not shown).

Fusion Assays—Cultures were grown in low calcium medium to early logarithmic phase. Mating partners were diluted to A_{600} 0.4 and mixed at a ratio of 1:1. Cells were mated on sterile 0.45 μm HA nitrocellulose filters (Millipore #HAWP02500) positioned on low calcium synthetic complete medium containing Noble agar. After 4 h at 30 °C, mating cells were resuspended in 1 ml of PBS containing 1 M sorbitol and 3.7% formaldehyde and fixed overnight at 4 °C. On the following day, cells were washed in 1 ml of PBS containing 1 M sorbitol and then sonicated (15-s bursts × 4 times) to disperse clumps. Cell membranes and nuclei were imaged by epifluorescence microscopy after staining with the lipophilic dye FM4–64 (Molecular Probes) and resuspension in mounting solution (70% glycerol, 30% PBS, 2% w/v *n*-propyl gallate) containing 0.0225 μg/ml DAPI (4′6-diamidino-Z-phenylindole). The frequency of mating pairs in which membrane and cell wall material were incompletely removed was determined for at least 300 zygotes.

Cell Death Assays—*S. cerevisiae* cultures were grown in synthetic complete medium at 30 °C to saturation and diluted 7-fold with 90 μl of fresh medium containing tunicamycin (2 μg/ml), miconazole (15 μM), or α-factor (50 μM). After 19 h of incubation at room temperature in flat-bottom 96-well dishes (BD Biosciences), the cells were resuspended with an equal volume of PBS containing 1 μg/ml propidium iodide. Live and dead cells were counted automatically using a 96-well flow cytometer FACSArray (BD Biosciences). At least 5000 cells were counted in each sample. *C. glabrata* cultures were grown

and assayed similarly, except the saturated cultures were diluted 49-fold into fresh medium, the concentration of tunicamycin was doubled, and the incubation time was shortened to 5.5 h.

RESULTS

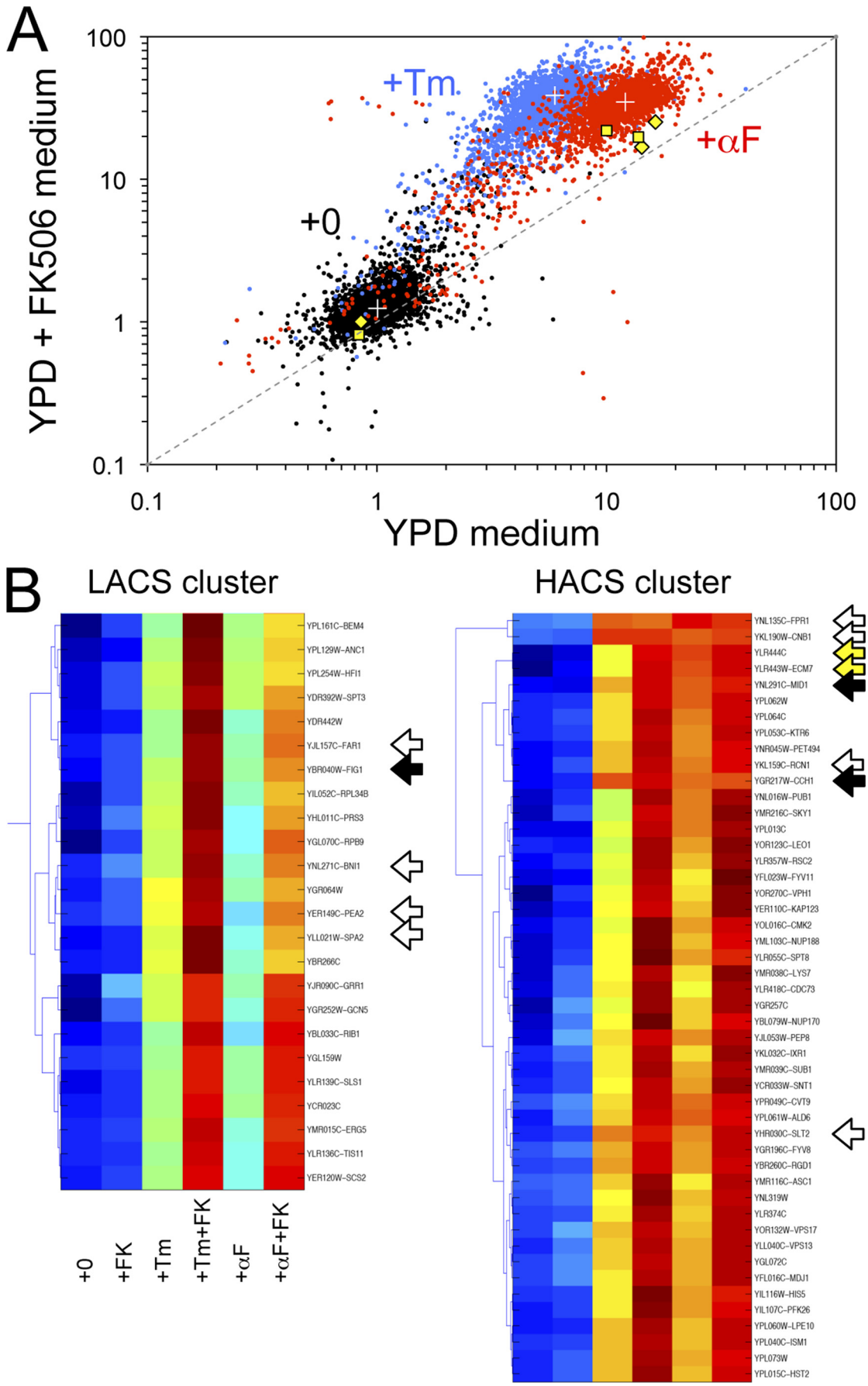
A Genome-wide Screen for Factors Involved in Ca^{2+} Uptake—Previous studies have shown that Cch1 and Mid1 are required for a HACS in *S. cerevisiae* that becomes activated in response to mating pheromones or tunicamycin especially in the presence of FK506, a specific inhibitor of the Ca^{2+}/calmodulin-dependent protein phosphatase calcineurin (7, 26, 27). To search for new subunits and regulators of HACS, we measured uptake of $^{45}Ca^{2+}$ tracer from the medium into 4848 different gene knock-out mutants of *S. cerevisiae* (18) after exposure to either α-factor mating pheromone, tunicamycin, or solvent control, each in the presence and absence of FK506. The vast majority of knock-out mutants exhibited a pattern of Ca^{2+} uptake that was indistinguishable from the wild type (Fig. 1*A* and supplemental Table 1). The HACS-deficient *cch1* and *mid1* mutants deviated significantly from the wild-type cells as expected (Fig. 1*A*, *yellow symbols*).

To identify new regulators or subunits of HACS, two statistical filters were used to eliminate mutants whose behavior was less than 2 S.D. distant from the population center or angle from the origin (see "Experimental Procedures"). The remaining mutants were subdivided into two groups. Group A mutants (187 in total) exhibited significantly lower or higher Ca^{2+} uptake in the absence of tunicamycin and α-factor and, therefore, represent stimulus-independent regulators of Ca^{2+} uptake. The Group A mutants were organized into phenotypic classes after hierarchical clustering of the data obtained from all six conditions. One cluster of 13 mutants (lines 1–13 of supplemental Table 1) exhibited very little Ca^{2+} accumulation in all conditions, probably due to low cell density. 9 of 10 mutants in another cluster with decreased Ca^{2+} uptake were deficient in subunits or regulators of the V-ATPase (lines 28–37 of supplemental Table 1). The V-ATPase is necessary for secondary Ca^{2+} transport into the vacuole, an organelle containing >90% of the total cell-associated Ca^{2+} (28). Interestingly, 86 (57%) of the remaining mutants in Group A (lines 38–187 of supplemental Table 1) exhibited significantly elevated Ca^{2+} accumulation in YPD medium plus FK506, suggesting spontaneous HACS activation in these mutants. This set of mutants was highly enriched for genes involved in vesicle-mediated transport (p value = 1.67E-22 using GO Term Finder) and other processes related to endomembrane trafficking and secretory protein biogenesis. Thirty of these 86 mutants also exhibited synthetic lethal or synthetic sick interactions with *cch1* or *mid1* mutations that destroy HACS, with *cnb1* mutations that destroy calcineurin, or with FK506 or cyclosporine compounds that inhibit calcineurin (29–35). Previous studies have demonstrated high Ca^{2+} influx and elevated $[Ca^{2+}]_{cyt}$ in *vps33* mutants, which have defects in sorting of proteins to the vacuole (36). Our dataset shows that many other *vps* mutants exhibit similarly high Ca^{2+} uptake in our conditions, including mutants lacking the GARP complex (*vps51*, *vps52*, *vps53*, *vps54*), a dynamin-like GTPase (*vps1*), clathrin (*chc1*, *clc1*, *swa2*), and a Na^+/H^+ exchanger (*nhx1*/*vps44*). Additionally, mutants that lack a furin-like protease (*kex2*) or the secretory pathway Ca^{2+} ATPase necessary for furin activity (*pmr1*) (37, 38) exhibited high Ca^{2+} uptake in the absence of the stimuli. We previously showed that *pmr1* mutants exhibit very high HACS activity (8). Taken together, these findings suggest that the activation of HACS (or perhaps another calcineurin-sensitive Ca^{2+} influx pathway in some cases) is a major response to defects in the secretory, endosomal, and vacuolar protein trafficking pathways.

A larger class of 572 mutants (Group B mutants, supplemental Table 1) had abnormal Ca^{2+} uptake only in the presence of tunicamycin or α-factor. After hierarchical clustering, several interesting subgroups were revealed. One cluster of 53 mutants (lines 1–53 in Group B in supplemental Table 1) exhibited little or no response to α-factor in the presence or absence of FK506 and normal responses to tunicamycin and tunicamycin plus FK506. This cluster contained *ste2*, *ste4*, *ste5*, *ste7*, *ste11*, *ste20*, *sir2*, *sir3* and other mutants that are known to be "sterile" or unresponsive to mating pheromones. A second cluster (Fig. 1*B*, *left*) contained *fig1*, *far1*, *bni1*, *spa2*, and *pea2* mutants, which are all deficient in a low affinity Ca^{2+} influx system (LACS) that is specifically induced in response to α-factor (15). A third cluster (Fig. 1*B*, *right*) exhibited altered HACS activity in response to both stimuli and contained the *cch1* and *mid1* mutants as well as the calcineurin-deficient *cnb1* and *rcn1* mutants and the *fpr1* mutant that lacks the FK506-binding protein necessary for inhibition of calcineurin by FK506. Among the six other mutants that clustered closest to *cch1* and *mid1*, three were flagged because of an equipment malfunction, and one was deficient in a mitochondrial function. The two remaining mutants in this subcluster (*ylr444c* and *ylr443w*) have overlapping deletions that inactivate the *ECM7* gene. An *ecm7* mutant of yeast was one of many mutants reported with general cell wall defects (39). Additionally, an *ecm7* mutant of the pathogenic yeast *C. glabrata* was one of several reported with hypersensitivity to the azole-class antifungal agent fluconazole (9). The Ecm7 proteins and their roles in Ca^{2+} uptake have not been previously studied.

Ecm7 Is Required for HACS—To test the importance of Ecm7 in HACS function, we re-examined the *ecm7* knock-out mutant of *S. cerevisiae* Ca^{2+} uptake in response to tunicamycin and α-factor. We also obtained an *ecm7* mutant of the human pathogen *C. glabrata* and measured Ca^{2+} uptake in response to tunicamycin and miconazole, an azole-class antifungal agent. In both species, the *ecm7* mutants exhibited striking defects in Ca^{2+} uptake that were quantitatively similar to those of *cch1* and *mid1* mutants (Fig. 2, *A* and *B*). In particular, the calcineurin-sensitive HACS activity was largely abolished in these mutants, leaving only the residual calcineurin-insensitive Ca^{2+} influx activities, including LACS. Thus, Ecm7 was required for HACS activity in these yeasts.

HACS can be activated very rapidly by a sudden elevation of environmental pH (4). To determine whether Ecm7 is also necessary for HACS activation by high pH shock, aequorin luminescence was used to monitor cytosolic-free Ca^{2+} concentrations ($[Ca^{2+}]_{cyt}$). After dilution with high pH culture medium, wild-type *S. cerevisiae* cells exhibited a large, rapid, and tran-


A
YPD + FK506 medium
YPD medium
100
10
1
0.1
0.1
1
10
100
+Tm
+αF
+0
B
LACS cluster
HACS cluster
YPL161C–BEM4
YPL129W–ANC1
YPL254W–HFI1
YDR392W–SPT3
YDR442W
YJL157C–FAR1
YBR040W–FIG1
YIL052C–RPL34B
YHL011C–PRS3
YGL070C–RPB9
YNL271C–BNI1
YGR064W
YER149C–PEA2
YLL021W–SPA2
YBR266C
YJR090C–GRR1
YGR252W–GCN5
YBL033C–RIB1
YGL159W
YLR139C–SLS1
YCR023C
YMR015C–ERG5
YLR136C–TIS11
YER120W–SCS2
+0
+FK
+Tm
+Tm+FK
+αF
+αF+FK
YNL135C–FPR1
YKL190W–CNB1
YLR444C
YLR443W–ECM7
YNL291C–MID1
YPL062W
YPL064C
YPL053C–KTR6
YNR045W–PET494
YKL159C–RCN1
YGR217W–CCH1
YNL016W–PUB1
YMR216C–SKY1
YPL013C
YOR123C–LEO1
YLR357W–RSC2
YFL023W–FYV11
YOR270C–VPH1
YER110C–KAP123
YOL016C–CMK2
YML103C–NUP188
YLR055C–SPT8
YMR038C–LYS7
YLR418C–CDC73
YGR257C
YBL079W–NUP170
YJL053W–PEP8
YKL032C–IXR1
YMR039C–SUB1
YCR033W–SNT1
YPR049C–CVT9
YPL061W–ALD6
YHR030C–SLT2
YGR196C–FYV8
YBR260C–RGD1
YMR116C–ASC1
YNL319W
YLR374C
YOR132W–VPS17
YLL040C–VPS13
YGL072C
YFL016C–MDJ1
YIL116W–HIS5
YIL107C–PFK26
YPL060W–LPE10
YPL040C–ISM1
YPL073W
YPL015C–HST2

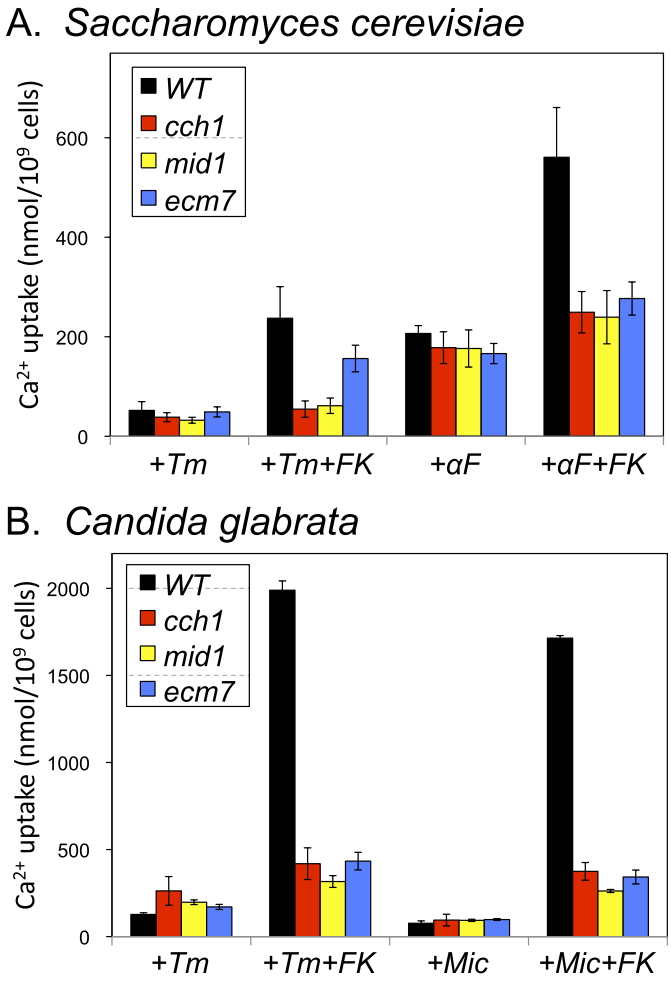


FIGURE 2. **Quantitative Ca^{2+} uptake experiments on *S. cerevisiae* and *C. glabrata* mutants lacking Cch1, Mid1, or Ecm7.** Log-phase cultures of BY4741 *S. cerevisiae* (*A*) and BG2 *C. glabrata* (*B*) were exposed to α-factor, tunicamycin (*Tm*), or miconazole (*Mic*) in the presence and absence of FK506 (*FK*) while growing in YPD medium containing tracer quantities of $^{45}CaCl_2$. *Bars* indicate the average of three independent determinations of Ca^{2+} uptake per billion cells (±S.D.). Similar results were obtained for independently generate *ecm7* mutants of *S. cerevisiae* W303-1A (data not shown).

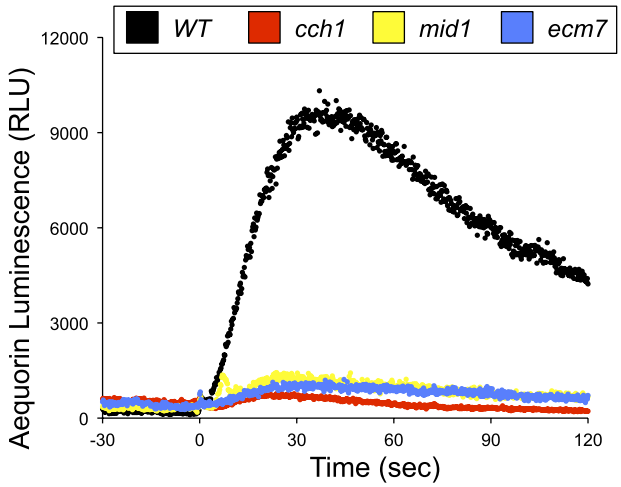


FIGURE 3. **Aequorin luminescence of *S. cerevisiae* mutants after high pH shock.** The *cch1*, *mid1*, and *ecm7* knock-out mutants and the wild-type W303-1A parent strain were transformed with aequorin expression plasmids and monitored for luminescence before and after dilution with pH 9 medium (at the 30-s time point). Relative luminescence units (*RLU*) were plotted after normalization for cell density.

sient elevation of $[Ca^{2+}]_{cyt}$, whereas the *ecm7* mutants exhibited no such response like the *cch1* and *mid1* mutants (Fig. 3). These findings show that, like Cch1 and Mid1, the previously uncharacterized protein Ecm7 is required for HACS activity in response to both fast and slow stimuli.

Ecm7, Mid1, and Cch1 Interact—To determine whether Ecm7 physically interacts with Cch1 or Mid1, we attempted co-immunoprecipitation experiments similar to those used previously to demonstrate a physical interaction between Cch1 and Mid1 (8). However, experiments using MYC-tagged and HA-tagged proteins failed to produce reliable evidence of a physical interaction in detergent and buffer conditions that preserve Cch1-Mid1 complexes (data not shown). As an alternative approach, we asked if Ecm7, Mid1, or Cch1 proteins depend on one another for expression or stability. For this experiment, the three proteins were each tagged at their C termini with the MYC epitope and analyzed by Western blotting of total cell lysates. Bands corresponding to MYC-tagged Ecm7, Mid1, and Cch1 had similar intensities in non-stimulated wild-type cells, suggesting they are expressed at roughly stoichiometric levels (not shown). Control experiments also showed the MYC tags did not interfere with HACS function. Interestingly, the abundance of Ecm7-MYC was slightly diminished in *mid1* and *cch1 mid1* mutant strains, and it was greatly diminished in these mutant strains after a 1-h chase with cycloheximide, an inhibitor of protein synthesis (Fig. 4, *top*). These findings suggest that Mid1 stabilizes Ecm7. Similarly, Mid1-MYC levels were reduced in *cch1* and *cch1 ecm7* mutant strains after cycloheximide chase (Fig. 4, *middle*), suggesting that Cch1 stabilizes Mid1. On the other hand, Cch1-MYC expression and stability were completely unaltered in *mid1 ecm7* double mutants and in the single mutants (Fig. 4, *bottom*), suggesting that Cch1 stability does not require Mid1 or Ecm7. Thus, Cch1 stabilized Mid1, and Mid1 stabilized Ecm7. Ecm7 did not stabilize Cch1 or Mid1. These findings suggest that Ecm7 directly or indirectly interacts with subunits of HACS and directly or indirectly regulates HACS through unknown mechanisms.

Ecm7 Is Homologous to the γ-Subunits of VGCCs and to Other Members of the PMP-22/EMP/MP20/Claudin Superfamily of Four-spanner Membrane Proteins—Ecm7 was predicted to contain four transmembrane helices with its N and C termini located within the cytoplasm (40). BLAST searches of genome databanks revealed orthologs of Ecm7 in the genomes of all *Saccharomyces* (yeasts) but no obvious orthologs in the genomes of other fungi, all of which contain orthologs of Cch1

FIGURE 1. **Genome-wide screen for mutants with altered Ca^{2+} uptake.** Individual mutants from the *S. cerevisiae* gene knock-out collection were exposed to tunicamycin (+*Tm*, *blue symbols*), α-factor (+*αF*, *red symbols*), or no stimulus (+*0*, *black symbols*) in the presence or absence FK506 (*FK*) while growing in YPD medium containing tracer quantities of $^{45}CaCl_2$. Total cell associated radioactivity was measured, and the normalized data were plotted (*A*) as described under "Experimental Procedures." *Yellow diamonds* indicate the *cch1* mutant, and *yellow squares* indicate the *mid1* mutant. After excluding mutants that failed statistical tests, the outliers were grouped by hierarchical clustering. Subclusters that contain known LACS- and HACS-deficient mutants were plotted (*B*). *Black*, *white*, and *yellow arrows* indicate mutants that lack transmembrane subunits, regulators, and putative new subunits of LACS and HACS, respectively.

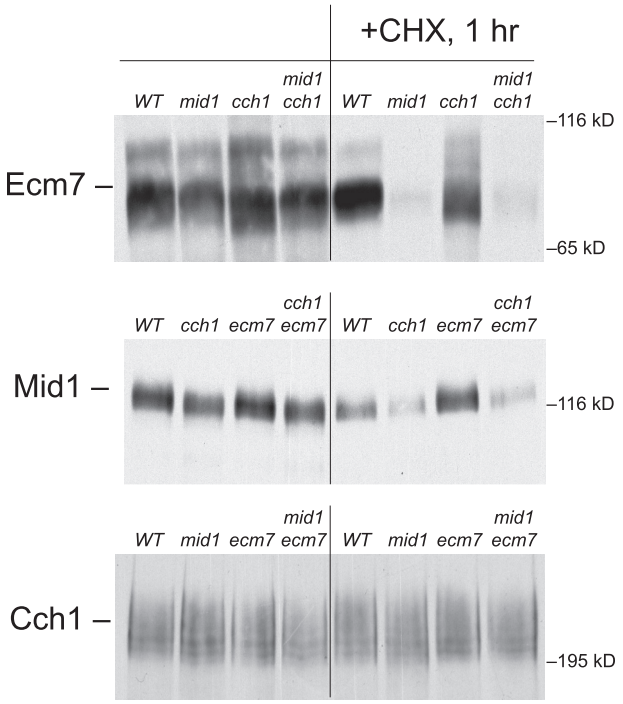


FIGURE 4. **Expression and stability of Ecm7, Mid1, and Cch1 proteins in *S. cerevisiae*.** The Ecm7, Mid1, and Cch1 proteins were tagged at their C termini in the indicated strains derived from W303-1A by chromosomal integration of the MYC epitope tag. Cells were grown to log phase with or without a 1-h exposure to cycloheximide (*CHX*), and then total cell lysates were fractionated by SDS-PAGE and analyzed by Western blotting. The *top*, *middle*, and *bottom panels* depict the Ecm7-MYC, Mid1-MYC, and Cch1-MYC proteins, respectively.

and Mid1. However, six supervised iterations of PSI-BLAST revealed numerous members of the "PMP-22/EMP/MP20/Claudin" superfamily (pfam00822) as significantly related to Ecm7. Several members of this superfamily in animals are known to function as stable γ-subunits of VGCCs that directly bind and regulate the Cch1-like α-subunits, whereas others regulate AMPA receptors, cell adhesion factors, and other factors (41). All members of this highly divergent superfamily are predicted to contain four transmembrane segments with cytoplasmic N and C termini and a consensus GLWGXCX_nC motif located in the first extracellular loop. Multiple sequence alignment of 17 Ecm7 orthologs from diverse yeasts revealed a highly conserved SLWGWCX_{15-27}C motif in the first extracellular loop, which strongly resembles the signature sequences of the PMP-22/EMP/MP20/Claudin superfamily. These findings suggest that Ecm7 is homologous to γ-subunits of VGCCs (and other members of the pfam00822 superfamily) and may function as a regulatory subunit of HACS.

Eleven Paralogs of Ecm7 in Yeast—In addition to Ecm7, similarity searches revealed 11 other members of the PMP-22/EMP/MP20/Claudin superfamily in the yeast proteome (Table 2). Eleven of the 12 were listed by the NCBI as members of a fungal-specific "Sur7/PalI" superfamily (pfam06687), which all contain four predicted transmembrane segments and a disulfide bond in the first extracellular loop. We classified the 12 proteins into six homology groups (classes A–F) based on multiple sequence alignment and phylogenetic analyses. Except for Ecm7 and Fig. 1, none of the single knock-out mutants of other

TABLE 2
Members of the PMP-22/EMP/MP20/Claudin and Sur7/Pall superfamilies in *S. cerevisiae*

		PSI-BLAST queries[a]				
	ORF/gene	Ecm7	Rim9	Sur7	PCC[b]	Class[c]
1	*YLR443w/ECM7*	1		4	0.983	A
2	*YML052w/SUR7*	3	6	1	0.998	B
3	*YNL194c*	4	6	1	0.993	B
4	*YDL222c/FMP45*	3	6	1	0.993	B
5	*YLR414c/PUN1*	2	7	2	0.997	C
6	*YLR413w*	3		4	0.955	C
7	*YKL187c*	2		4	0.953	C
8	*YBR040w/FIG1*	4		5	0.996	D
9	*YMR063w/RIM9*		1	5	0.995	E
10	*YFR012w*		2	4	0.993	E
11	*YOL019w*		2	4	0.979	E
12	*YML066c/SMA2*				0.993	F

[a] Numbers indicate the iteration of PSI-BLAST at which each gene was found to be significant (E-value < 0.001) using the query sequence indicated.
[b] Pearson correlation coefficients of each parameterized protein sequence to the median of sequences 1–11.
[c] Arbitrary class designations based on phylogenetic trees from multiple sequence alignments.

superfamily members exhibited abnormal Ca^{2+} accumulation in our screening conditions. Functional redundancy among these proteins may have obscured potential roles in Ca^{2+} influx. Alternatively, they may promote Ca^{2+} influx in other conditions or promote processes unrelated to Ca^{2+} transport similar to the claudins in animals, which typically function in cell-cell adhesion (16).

Roles of Cch1, Mid1, and Ecm7 in Mating—Although HACS becomes activated in response to mating pheromones, the roles of Cch1, Mid1, and Ecm7 in mating have not been carefully studied. To investigate the role of these factors in mating, we performed patch matings of the *cch1*, *mid1*, and *ecm7* single mutants both with wild-type cells and in combination. Mating efficiencies were noticeably diminished in *cch1* × *cch1* and *mid1* × *mid1* matings but not *ecm7* × *ecm7* matings or other mutant combinations in low calcium medium (Fig. 5). The defects observed in *cch1* × *cch1* and *mid1* × *mid1* matings were also evident in medium containing 20 mM $CaCl_2$ (data not shown) and were not further exacerbated by the loss of Fig. 1, another PMP-22/EMP/MP20/Claudin superfamily member that is essential for LACS in one or both partners (Fig. 5). Therefore, the role of Cch1 and Mid1 in mating may be independent of extracellular Ca^{2+} and not overlapping with LACS. The observation that *cch1* × *mid1* matings were much more efficient than the *cch1* × *cch1* or *mid1* × *mid1* matings suggests that HACS is not required in the haploid cells before mating. The findings reveal important post-zygotic roles for Cch1 and Mid1 in mating that may be independent of Ca^{2+} and Ecm7.

To examine the mating defects of *cch1* and *mid1* mutants in more detail, the lipophilic probe FM4–64 was used to stain the plasma membranes of cells in the process of mating. In *WT* × *WT* matings, less than 5% of zygotes failed to completely remove cell wall and plasma membrane material. The frequency of abnormal zygotes rose to >40% in *cch1* × *cch1* and *mid1* × *mid1* matings (Fig. 5, *bottom*). Interestingly, *fig1* × *fig1* matings exhibited ~40% abnormal zygotes, as seen previously (42), although the abnormal fusion did not detectably alter mating efficiency. The fusion defects of Fig. 1-deficient matings were increased even more by the additional losses of Cch1 or

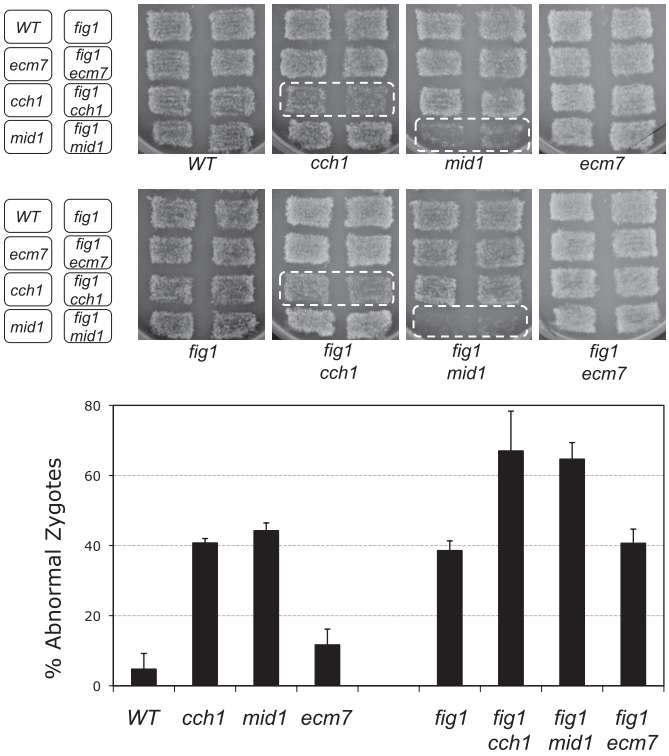


FIGURE 5. **Mating and fusion efficiencies of HACS-deficient mutants.** *Top*, the indicated single and double mutants of strains BY4741 and BY4742 were allowed to mate for 4 h in low-calcium medium and then replica-plated to diploid selection medium. After 1 day of re-growth, relative mating efficiencies were visible. Similar results were obtained using high-calcium medium (not shown). *Bottom*, homozygous matings of the indicated strains were performed for a brief period of time and then stained with FM4–64. The zygotes were then counted as abnormal if cell wall or cell membrane material persisted within their fusion zones and are plotted as a percentage of the total number of zygotes. *Bars* indicate the averages of three-seven independent experiments (±S.D.).

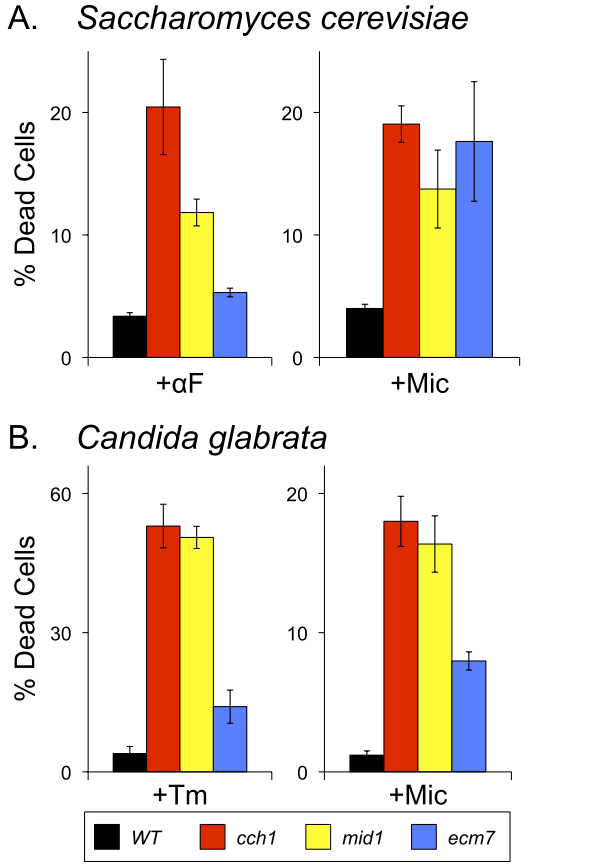


FIGURE 6. **Stimulus-dependent death of HACS-deficient cells.** The indicated mutants of *S. cerevisiae* (*A*) and *C. glabrata* (*B*) were grown in synthetic complete medium and exposed to α-factor (+α*F*), tunicamycin (+*Tm*), or miconazole (+*Mic*) as indicated for ~5 h. Live and dead cells were counted by flow cytometry after staining with propidium iodide. *Bars* represent the average of three independent cultures (±S.D.).

Mid1 (Fig. 5, *bottom*). In contrast, the *ecm7* × *ecm7* matings with or without Fig. 1 more closely resembled the control matings than the Cch1- and Mid1-deficient matings. These findings suggest that Fig. 1 and Cch1/Mid1 promote cell fusion during mating by two different mechanisms and that all these proteins may function independent of Ecm7. One possible explanation for these Ecm7-independent effects of Cch1 and Mid1 in mating is that another member of the PMP-22/EMP/MP20/Claudin superfamily in *S. cerevisiae* may function redundantly with Ecm7.

Anti-fungicidal Effects of Cch1, Mid1, and Ecm7—Prolonged exposure to mating pheromones, tunicamycin, or azole-class antifungal agents under the appropriate culture conditions can kill *cch1* and *mid1* mutant cells but not wild-type cells of several yeast species (6, 9, 27). The *ecm7* mutant of *S. cerevisiae* also died in response to miconazole and tunicamycin at a rate similar to the *cch1* and *mid1* mutants (Fig. 6*A* and data not shown) but did not exhibit an elevated rate of cell death during exposure to α-factor (Fig. 6*A*). The *cch1*, *mid1*, and *ecm7* mutants of *C. glabrata* all died at elevated rates when exposed to tunicamycin or miconazole (Fig. 6*B*). Thus, all three components of HACS were important for resistance of these yeasts to the fungicidal effects of tunicamycin and miconazole.

DISCUSSION

HACS Structure and the Ancestral VGCC—The experimental and phylogenetic findings reported here all support the hypothesis that Ecm7 is a regulatory subunit of HACS that is homologous to γ-subunits of VGCCs. Although we have not yet localized Ecm7 or co-purified it with Cch1 or Mid1, Ecm7 was stabilized by Mid1, and Mid1 was stabilized by Cch1 in unstimulated cells, which suggests physical interactions between the proteins. Mutants lacking *ECM7* behaved much like *cch1* and *mid1* null mutants in a variety of quantitative tests, including Ca^{2+} uptake in response to tunicamycin and α-factor and $[Ca^{2+}]_{cyt}$ elevation in response to high pH shock. Curiously, *ecm7* null mutants exhibited weaker phenotypes than *cch1* and *mid1* mutants in assays of cell survival during the response to antifungal drugs and cell fusion during sexual conjugation. Ecm7-deficient mutants of *S. cerevisiae* were not recovered in the previous genetic screens that yielded *cch1* and *mid1* mutants (2, 6) possibly because residual HACS activity may exist in *ecm7* mutants. Recently, *ecm7* mutants of *C. glabrata* were recovered with *mid1* and *cch1* mutants in a genetic screen for sensitivity to the azole-class antifungal agent fluconazole, but once again, the *ecm7* phenotypes were less pronounced than those of *cch1* and *mid1* mutants (9). These findings together suggest that HACS structure and regulation may be conserved among the yeasts.

ECM7 was originally identified in a screen of transposon-induced mutants that exhibit defects in the cell wall (39). However, cell wall defects have not been observed in *ecm7*, *mid1*, or *cch1* null mutants. Remarkably, across hundreds of other conditions employed in a chemical genomics screen, the phenotypes of *ecm7* null mutants were most closely correlated to those of *mid1* null mutants, and vice versa (43). Thus, the available genetic data indicate that Ecm7, Mid1, and Cch1 largely function together, although they do not rule out additional independent functions or additional components of the system.

Ecm7 is homologous to γ-subunits of human VGCCs and other members of the PMP-22/EMP/MP20/Claudin superfamily (pfam00822) and the Sur7/PalI superfamily (pfam06687), which we propose to be merged into a single superfamily. Of 11 paralogs of Ecm7 in yeast, only a few have been characterized to date. Fig. 1 has been defined previously as a regulator or component of a LACS that is activated specifically in response to mating pheromones (15). Fig. 1 is strongly induced during the response to mating pheromones and also promotes cell fusion during mating (42). Sma2 is induced during sporulation and is necessary for formation of spore cell membrane (44). The genes encoding Sma2 and Ecm7 are adjacent to one another in the genomes of many yeasts (45), which may indicate they derive from a tandem gene duplication event in an ancestral yeast species. It will be interesting to test if Sma2 can replace Ecm7 function in some circumstances. The three class B proteins in the superfamily (Sur7, YDL222w, YNL194c) all localize to punctate subdomains of the plasma membrane and seem to modulate sphingolipid biosynthesis and endocytosis (46). Rim9 and other membrane proteins are necessary for ambient pH sensing and sporulation (47, 48). The other paralogs of Ecm7 in yeast remain uncharacterized. When the diverse functions of animal PMP-22/EMP/MP20/Claudin superfamily proteins are also considered, the general function of these proteins seems to involve the trafficking, localization, organization, and/or regulation of various membrane-bound transporters and enzymes, including VGCCs and other types of ion channels.

Cch1 is clearly homologous to the catalytic α-subunits of human VGCCs and retains strong sequence conservation particularly in the active site and transmembrane regions. Thus, the ancestral VGCC may have contained both α- and γ-subunits. Some mammalian VGCCs also contain peripheral β-subunits (3), which are not strongly conserved at the sequence level and are not found outside of animals and their closest relatives (49). Most mammalian VGCCs also contain α2δ-subunits (3), which typically contain an N-terminal signal peptide, a large extracellular domain containing 12 or more cysteine residues, and a C-terminal GPI-anchor (50). Homology searches reveal homologs of α2δ-subunits in the genomes of animals but not fungi. Nevertheless, many of their structural features are evident in Mid1-related proteins, which can be found only in the genomes of fungi (51).[3] Therefore, Mid1 may be structurally and functionally analogous to α2δ-subunits if not demonstrably homologous. Therefore, VGCCs may have been heterotrimers of Cch1/α-, Mid1/α2δ-, and Ecm7/γ-like subunits in the common ancestor of animals and fungi. Limiting quantities of Ecm7 may prevent huge increases in HACS activity in *S. cerevisiae* cells overexpressing both Cch1 and Mid1 (52).

HACS Regulation—High pH shock rapidly stimulates the Ca^{2+} influx activity of HACS (4). The high pH shock may activate HACS directly through deprotonation of residues involved in ion conduction or channel gating (53). Alternatively, the high pH shock may transiently depolarize the cell membrane and promote opening of HACS similar to the activation mechanism of VGCCs in animal cells. The rapid activation of HACS by high pH shock was independent of calcineurin (data not shown), suggesting that phosphorylation and dephosphorylation of Cch1 or other subunits may not be necessary for HACS regulation in these conditions. On the other hand, tunicamycin and mating pheromones appear to activate HACS with much slower kinetics through a process that is sensitive to the activation of calcineurin and dependent on the activation of MAP kinases such as Slt2 and Fus3 (8). MAP kinase activation leads to phosphorylation of Cch1 at unknown sites in the absence of calcineurin, and subsequent calcineurin activation leads to dephosphorylation of Cch1 (8). Whether these stimuli-dependent modifications of Cch1 can occur in the absence of Mid1 or Ecm7 is not yet clear. Additionally, the physiological significance of these modifications has not been established with appropriate mutants, so the possibility that MAP kinases and calcineurin regulate HACS indirectly through the effects on transmembrane potential cannot be excluded.

Our measurements of Ca^{2+} uptake into thousands of different gene knock-out mutants of yeast provide clues about the physiological phenomena that might regulate HACS. For example, mutations affecting various aspects of the endomembrane trafficking system, particularly those associated with the Golgi complex and trans-Golgi network, activated HACS in the absence of external stimuli. Interestingly, many of these HACS-activating mutants rely on HACS and calcineurin subunits for growth. Because HACS and calcineurin are largely inactive during vegetative growth of unstressed wild-type cells (54), our genome-wide analysis of HACS regulation suggests that a major function of the calcium signaling network is to compensate for various primary stresses or defects in the endomembrane trafficking system. Compounds such as tunicamycin, dithiothreitol, and azoles-class antifungal agents may phenocopy some of these HACS-activating mutations. Mating pheromones may also generate similar kinds of membrane stresses, and all these conditions may be ameliorated to some degree by the activation of HACS and calcineurin.

Other Implications—Several compounds with unknown targets and consequences have been linked to the activation HACS or calcineurin in *S. cerevisiae*. For instance, the antiarrhythmic drug amiodarone and the anticancer drug tamoxifen have been shown to alter Ca^{2+} homeostasis and to kill yeast cells in appropriate conditions (55–58). Based on the genome-wide analysis presented here, membrane trafficking factors or membranes themselves may be plausible targets of these compounds in yeasts. The identification of membrane-active toxins, inhibitors, and antifungal agents may be facilitated by quantitative measurements of Ca^{2+} uptake in yeasts.

[3] K. W. Cunningham, unpublished observations.

Although it is clear that HACS and calcineurin prevent death of yeast cells exposed to common antifungal agents, many important questions remain to be answered. For instance, the specific targets of calcineurin that promote fungal cell survival during endomembrane stresses and mating responses have not been identified. Additionally, it is not yet clear whether endomembrane stresses and mating pheromones cause electrochemical depolarization of the plasma membrane as part of the HACS activation process, as would be expected from the well known mechanisms of VGCC activation in animal cells. Drugs that target other components of the calcium signaling network may be more useful as antifungal co-drugs than the existing inhibitors of calcineurin, which also disrupt processes in the host. Deeper analyses of the genome-wide data produced here may reveal additional factors in the calcium signaling network in *S. cerevisiae* and provide new insights into quality control pathways that respond to endomembrane stresses.

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REFERENCES

1. Fischer, M., Schnell, N., Chattaway, J., Davies, P., Dixon, G., and Sanders, D. (1997) *FEBS Lett.* **419,** 259–262
2. Paidhungat, M., and Garrett, S. (1997) *Mol. Cell. Biol.* **17,** 6339–6347
3. Catterall, W. A. (2000) *Annu. Rev. Cell Dev. Biol.* **16,** 521–555
4. Viladevall, L., Serrano, R., Ruiz, A., Domenech, G., Giraldo, J., Barceló, A., and Ariño, J. (2004) *J. Biol. Chem.* **279,** 43614–43624
5. Iida, H., Yagawa, Y., and Anraku, Y. (1990) *J. Biol. Chem.* **265,** 13391–13399
6. Iida, H., Nakamura, H., Ono, T., Okumura, M. S., and Anraku, Y. (1994) *Mol. Cell. Biol.* **14,** 8259–8271
7. Muller, E. M., Locke, E. G., and Cunningham, K. W. (2001) *Genetics* **159,** 1527–1538
8. Locke, E. G., Bonilla, M., Liang, L., Takita, Y., and Cunningham, K. W. (2000) *Mol. Cell. Biol.* **20,** 6686–6694
9. Kaur, R., Castaño, I., and Cormack, B. P. (2004) *Antimicrob. Agents Chemother.* **48,** 1600–1613
10. Liu, M., Du, P., Heinrich, G., Cox, G. M., and Gelli, A. (2006) *Eukaryot. Cell* **5,** 1788–1796
11. Reedy, J. L., Filler, S. G., and Heitman, J. (2010) *Fungal Genet. Biol.* **47,** 107–116
12. Hong, M. P., Vu, K., Bautos, J., and Gelli, A. (2010) *J. Biol. Chem.* **285,** 10951–10958
13. Stie, J., and Fox, D. (2008) *Eukaryot. Cell* **7,** 177–186
14. Bonilla, M., and Cunningham, K. W. (2003) *Mol. Biol. Cell* **14,** 4296–4305
15. Muller, E. M., Mackin, N. A., Erdman, S. E., and Cunningham, K. W. (2003) *J. Biol. Chem.* **278,** 38461–38469
16. Van Itallie, C. M., and Anderson, J. M. (2006) *Annu. Rev. Physiol.* **68,** 403–429
17. Wallis, J. W., Chrebet, G., Brodsky, G., Rolfe, M., and Rothstein, R. (1989) *Cell* **58,** 409–419
18. Giaever, G., Chu, A. M., Ni, L., Connelly, C., Riles, L., Véronneau, S., Dow, S., Lucau-Danila, A., Anderson, K., André, B., Arkin, A. P., Astromoff, A., El-Bakkoury, M., Bangham, R., Benito, R., Brachat, S., Campanaro, S., Curtiss, M., Davis, K., Deutschbauer, A., Entian, K. D., Flaherty, P., Foury, F., Garfinkel, D. J., Gerstein, M., Gotte, D., Güldener, U., Hegemann, J. H., Hempel, S., Herman, Z., Jaramillo, D. F., Kelly, D. E., Kelly, S. L., Kötter, P., LaBonte, D., Lamb, D. C., Lan, N., Liang, H., Liao, H., Liu, L., Luo, C., Lussier, M., Mao, R., Menard, P., Ooi, S. L., Revuelta, J. L., Roberts, C. J., Rose, M., Ross-Macdonald, P., Scherens, B., Schimmack, G., Shafer, B., Shoemaker, D. D., Sookhai-Mahadeo, S., Storms, R. K., Strathern, J. N., Valle, G., Voet, M., Volckaert, G., Wang, C. Y., Ward, T. R., Wilhelmy, J., Winzeler, E. A., Yang, Y., Yen, G., Youngman, E., Yu, K., Bussey, H., Boeke, J. D., Snyder, M., Philippsen, P., Davis, R. W., and Johnston, M. (2002) *Nature* **418,** 387–391
19. Longtine, M. S., McKenzie, A., 3rd, Demarini, D. J., Shah, N. G., Wach, A., Brachat, A., Philippsen, P., and Pringle, J. R. (1998) *Yeast* **14,** 953–961
20. Sherman, F., Hicks, J. B., and Fink, G. R. (1986) *Methods in Yeast Genetics*, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY
21. Rousseeuw, P. J., and Van Driessen, K. (1999) *Technometrics* **41,** 212–223
22. Penny, I. I. (1996) *Applied Statistics* **45,** 73–81
23. Cunningham, K. W., and Fink, G. R. (1994) *J. Cell Biol.* **124,** 351–363
24. Altschul, S. F., Madden, T. L., Schäffer, A. A., Zhang, J., Zhang, Z., Miller, W., and Lipman, D. J. (1997) *Nucleic Acids Res.* **25,** 3389–3402
25. Ward, J. M. (2001) *Bioinformatics* **17,** 560–563
26. Matheos, D. P., Kingsbury, T. J., Ahsan, U. S., and Cunningham, K. W. (1997) *Genes Dev.* **11,** 3445–3458
27. Bonilla, M., Nastase, K. K., and Cunningham, K. W. (2002) *EMBO J.* **21,** 2343–2353
28. Dunn, T., Gable, K., and Beeler, T. (1994) *J. Biol. Chem.* **269,** 7273–7278
29. Conboy, M. J., and Cyert, M. S. (2000) *Mol. Biol. Cell* **11,** 2429–2443
30. Lesage, G., Sdicu, A. M., Ménard, P., Shapiro, J., Hussein, S., and Bussey, H. (2004) *Genetics* **167,** 35–49
31. Tong, A. H., Lesage, G., Bader, G. D., Ding, H., Xu, H., Xin, X., Young, J., Berriz, G. F., Brost, R. L., Chang, M., Chen, Y., Cheng, X., Chua, G., Friesen, H., Goldberg, D. S., Haynes, J., Humphries, C., He, G., Hussein, S., Ke, L., Krogan, N., Li, Z., Levinson, J. N., Lu, H., Ménard, P., Munyana, C., Parsons, A. B., Ryan, O., Tonikian, R., Roberts, T., Sdicu, A. M., Shapiro, J., Sheikh, B., Suter, B., Wong, S. L., Zhang, L. V., Zhu, H., Burd, C. G., Munro, S., Sander, C., Rine, J., Greenblatt, J., Peter, M., Bretscher, A., Bell, G., Roth, F. P., Brown, G. W., Andrews, B., Bussey, H., and Boone, C. (2004) *Science* **303,** 808–813
32. Parsons, A. B., Brost, R. L., Ding, H., Li, Z., Zhang, C., Sheikh, B., Brown, G. W., Kane, P. M., Hughes, T. R., and Boone, C. (2004) *Nat. Biotechnol.* **22,** 62–69
33. Dixon, S. J., Fedyshyn, Y., Koh, J. L., Prasad, T. S., Chahwan, C., Chua, G., Toufighi, K., Baryshnikova, A., Hayles, J., Hoe, K. L., Kim, D. U., Park, H. O., Myers, C. L., Pandey, A., Durocher, D., Andrews, B. J., and Boone, C. (2008) *Proc. Natl. Acad. Sci. U.S.A.* **105,** 16653–16658
34. Fiedler, D., Braberg, H., Mehta, M., Chechik, G., Cagney, G., Mukherjee, P., Silva, A. C., Shales, M., Collins, S. R., van Wageningen, S., Kemmeren, P., Holstege, F. C., Weissman, J. S., Keogh, M. C., Koller, D., Shokat, K. M., and Krogan, N. J. (2009) *Cell* **136,** 952–963
35. Costanzo, M., Baryshnikova, A., Bellay, J., Kim, Y., Spear, E. D., Sevier, C. S., Ding, H., Koh, J. L., Toufighi, K., Mostafavi, S., Prinz, J., St Onge, R. P., VanderSluis, B., Makhnevych, T., Vizeacoumar, F. J., Alizadeh, S., Bahr, S., Brost, R. L., Chen, Y., Cokol, M., Deshpande, R., Li, Z., Lin, Z. Y., Liang, W., Marback, M., Paw, J., San Luis, B. J., Shuteriqi, E., Tong, A. H., van Dyk, N., Wallace, I. M., Whitney, J. A., Weirauch, M. T., Zhong, G., Zhu, H., Houry, W. A., Brudno, M., Ragibizadeh, S., Papp, B., Pál, C., Roth, F. P., Giaever, G., Nislow, C., Troyanskaya, O. G., Bussey, H., Bader, G. D., Gingras, A. C., Morris, Q. D., Kim, P. M., Kaiser, C. A., Myers, C. L., Andrews, B. J., and Boone, C. (2010) *Science* **327,** 425–431
36. Miseta, A., Fu, L., Kellermayer, R., Buckley, J., and Bedwell, D. M. (1999) *J. Biol. Chem.* **274,** 5939–5947
37. Fuller, R. S., Brake, A., and Thorner, J. (1989) *Proc. Natl. Acad. Sci. U.S.A.* **86,** 1434–1438
38. Antebi, A., and Fink, G. R. (1992) *Mol. Biol. Cell* **3,** 633–654
39. Lussier, M., White, A. M., Sheraton, J., di Paolo, T., Treadwell, J., Southard, S. B., Horenstein, C. I., Chen-Weiner, J., Ram, A. F., Kapteyn, J. C., Roemer, T. W., Vo, D. H., Bondoc, D. C., Hall, J., Zhong, W. W., Sdicu, A. M., Davies, J., Klis, F. M., Robbins, P. W., and Bussey, H. (1997) *Genetics* **147,** 435–450
40. Kim, H., Melén, K., and von Heijne, G. (2003) *J. Biol. Chem.* **278,** 10208–10213
41. Chu, P. J., Robertson, H. M., and Best, P. M. (2001) *Gene* **280,** 37–48
42. Erdman, S., Lin, L., Malczynski, M., and Snyder, M. (1998) *J. Cell Biol.* **140,** 461–483

43. Hillenmeyer, M. E., Fung, E., Wildenhain, J., Pierce, S. E., Hoon, S., Lee, W., Proctor, M., St Onge, R. P., Tyers, M., Koller, D., Altman, R. B., Davis, R. W., Nislow, C., and Giaever, G. (2008) *Science* **320,** 362–365
44. Rabitsch, K. P., Tóth, A., Gálová, M., Schleiffer, A., Schaffner, G., Aigner, E., Rupp, C., Penkner, A. M., Moreno-Borchart, A. C., Primig, M., Esposito, R. E., Klein, F., Knop, M., and Nasmyth, K. (2001) *Curr. Biol.* **11,** 1001–1009
45. Gordon, J. L., Byrne, K. P., and Wolfe, K. H. (2009) *PLoS Genet.* **5,** e1000485
46. Young, M. E., Karpova, T. S., Brügger, B., Moschenross, D. M., Wang, G. K., Schneiter, R., Wieland, F. T., and Cooper, J. A. (2002) *Mol. Cell. Biol.* **22,** 927–934
47. Li, W., and Mitchell, A. P. (1997) *Genetics* **145,** 63–73
48. Tréton, B., Blanchin-Roland, S., Lambert, M., Lépingle, A., and Gaillardin, C. (2000) *Mol. Gen. Genet.* **263,** 505–513
49. de Mendoza, A., Suga, H., and Ruiz-Trillo, I. (2010) *BMC Evol. Biol.* **10,** 93
50. Davies, A., Kadurin, I., Alvarez-Laviada, A., Douglas, L., Nieto-Rostro, M., Bauer, C. S., Pratt, W. S., and Dolphin, A. C. (2010) *Proc. Natl. Acad. Sci. U.S.A.* **107,** 1654–1659
51. Plaine, A., Walker, L., Da Costa, G., Mora-Montes, H. M., McKinnon, A., Gow, N. A., Gaillardin, C., Munro, C. A., and Richard, M. L. (2008) *Fungal Genet. Biol.* **45,** 1404–1414
52. Iida, K., Tada, T., and Iida, H. (2004) *FEBS Lett.* **576,** 291–296
53. Chen, X. H., Bezprozvanny, I., and Tsien, R. W. (1996) *J. Gen. Physiol.* **108,** 363–374
54. Yoshimoto, H., Saltsman, K., Gasch, A. P., Li, H. X., Ogawa, N., Botstein, D., Brown, P. O., and Cyert, M. S. (2002) *J. Biol. Chem.* **277,** 31079–31088
55. Courchesne, W. E., and Ozturk, S. (2003) *Mol. Microbiol.* **47,** 223–234
56. Gupta, S. S., Ton, V. K., Beaudry, V., Rulli, S., Cunningham, K., and Rao, R. (2003) *J. Biol. Chem.* **278,** 28831–28839
57. Muend, S., and Rao, R. (2008) *FEMS Yeast Res.* **8,** 425–431
58. Parsons, A. B., Lopez, A., Givoni, I. E., Williams, D. E., Gray, C. A., Porter, J., Chua, G., Sopko, R., Brost, R. L., Ho, C. H., Wang, J., Ketela, T., Brenner, C., Brill, J. A., Fernandez, G. E., Lorenz, T. C., Payne, G. S., Ishihara, S., Ohya, Y., Andrews, B., Hughes, T. R., Frey, B. J., Graham, T. R., Andersen, R. J., and Boone, C. (2006) *Cell* **126,** 611–625