Rhb1 Regulates the Expression of Secreted Aspartic Protease 2 through the TOR Signaling Pathway in Candida albicans

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Candida albicans is a major fungal pathogen in humans. In C. albicans, secreted aspartyl protease 2 (Sap2) is the most highly expressed secreted aspartic protease in vitro and is a virulence factor. Recent research links the small GTPase Rhb1 to C. albicans target of rapamycin (TOR) signaling in response to nitrogen availability. The results of this study show that Rhb1 is related to cell growth through the control of SAP2 expression when protein is the major nitrogen source. This process involves various components of the TOR signaling pathway, including Tor1 kinase and its downstream effectors. TOR signaling not only controls Sap2 transcription but also affects Sap2 protein levels, possibly through general amino acid control. DNA microarray analysis identifies other target genes downstream of Rhb1 in addition to SAP2. These findings provide new insight into nutrients, Rhb1-TOR signaling, and expression of C. albicans virulence factor.

Candida albicans is a major opportunistic fungal pathogen in humans, and it inhabits the skin and mucosal surfaces of healthy people (12). In immunocompromised patients such as those undergoing organ transplantation or chemotherapy or those with AIDS, C. albicans is responsible for a number of life-threatening infections with considerable morbidity and mortality (54). Several virulence factors contribute to C. albicans infection, including the expression of adhesins, yeast-to-hypha morphogenesis, phenotypic switching, and secreted hydrolytic activities (13).

Secreted aspartyl proteases (Saps) encoded by 10 members of the SAP gene family, SAP1 to SAP10 (50), are among the hydrolytic factors of C. albicans virulence. These genes are differentially expressed during various stages of C. albicans-host interaction, and each Sap protein possesses unique enzymatic characteristics and substrate specificities (50). Sap2 is the most highly expressed secreted protease in vitro and is capable of digesting human albumin, hemoglobin, keratin, and secreted immunoglobulin A (33). Sap2 is believed to allow C. albicans to destroy host barriers through the degradation of human proteins, followed by deep penetration into tissues or the bloodstream. In addition, Sap2 digests extracellular proteins into oligopeptides that can be taken up by oligopeptide transporters encoded by the OPT gene family (59). Therefore, Sap2 may also be critical for cell growth in the human host and may enable the use of host proteins as a nitrogen source. A C. albicans strain that lacks SAP2 loses its virulence in a murine model of infection (34). However, using URA3 as a marker during the construction of C. albicans mutants can make it difficult to interpret the resulting phenotypes (4, 11, 16, 37, 65). When URA3 assessment is eliminated from strain construction, Sap1 to Sap6 do not seem to be required for C. albicans invasion into reconstituted human epithelia (39). Sap1 to -6 played limited roles in C. albicans virulence and the host immune response in a murine model of disseminated infection (17). These studies showed that the roles of Sap2 and other Sap proteins in vivo still need to be clarified.

In fungi, the target of rapamycin (TOR) signaling pathway plays a key role in controlling different cellular processes in response to multiple environmental cues, including nutrient availability and stress (55, 61). The central component of this pathway is Tor, a serine/threonine protein kinase. Saccharomyces cerevisiae contains 2 TOR genes encoding 2 closely related kinases, Tor1 and Tor2. In association with a subset of proteins, including Kog1, Lst8, and Tco89, Tor1 or Tor2 forms TOR complex 1 (TORC1) (15, 41, 58, 79). Tor2 can also associate with another subset of proteins (e.g., Avo1, Avo2, Lst8, and Bit2) to form TOR complex 2 (TORC2) (24, 41, 58, 79). Although TORC1 and TORC2 share some functions, each complex controls distinct cell processes in response to environmental signals (41, 61).

In S. cerevisiae, TORC1 is rapamycin sensitive (61). Genetic analysis reveals that TORC1 functionally interacts with genes involved in actin polarization and membrane trafficking (2). TORC1 is also involved in nitrogen catabolite repression (NCR) through its regulation of 2 GATA-type transcriptional factors, Gat1 and Gln3 (7, 9, 18, 27, 35, 45, 46, 61, 62). In the presence of good nitrogen sources, Gln3 and Gat1 are restricted to the cytosol, leading to inactivation of the NCR genes. Similar expression patterns of NCR genes during nitrogen deprivation also appear in cells treated with rapamycin (14). The TORC2 protein complex regulates the polarization of the actin cytoskeleton in a rapamycin-insensitive manner (41). TORC2 is also involved in controlling cell wall integrity and receptor endocytosis (23). In the fission yeast Schizosaccharomyces pombe, loss of Tor2 function activates genes that are also induced during nitrogen starvation (49). Only 1 TOR gene, TOR1, has been identified in C. albicans (20). A recent study suggests that the C. albicans Tor1 kinase is involved in cell-cell adhesion and biofilm formation in nutrient-poor Spider medium but not under other tested conditions (6). Cell adhesion and biofilm formation are mediated by controlling the expression of the adhesion genes ALS1, ALS3, and HWP1 through Tor1 (6).
C. albicans, Gln3 and Gat1 are also regulate nitrogen metabolism and may function downstream of Tor1 kinase (40).

Recent research links the small GTPase Rhb1 to Tor1 signaling in C. albicans (72). Rhb1 was first identified in mammals and functions as a positive regulator of mammalian Tor (mTOR) kinases (42). In humans, GTP binding activates the homolog of C. albicans Rhb1 (named Rheb), and Rheb-GTP in turn stimulates the kinase activity of mammalian TORC1 (mTORC1) to trigger downstream signaling cascades (42). In fission yeast, rhb1-null mutants show arrested cell growth and division with a terminal phenotype similar to that caused by nitrogen starvation (44). Rhb1 depletion induces the expression of جنيn1 ' and mei2', which are normally induced by nitrogen starvation (44). In C. albicans, Rhb1-TOR is involved in nitrogen starvation-induced morphogenesis, likely by controlling the expression of MEP2 (72). MEP2 is a permease and ammonium sensor (10, 21). The expression of MEP2 is upregulated in nitrogen-limited conditions and is controlled by C. albicans Gln3 and Gat1 (21). In addition, gln3 and gat1 deletion mutants exhibit reduced sensitivity to rapamycin (40), suggesting that these transcriptional regulators may also be involved in TOR signaling in C. albicans. In the presence of protein and low nitrogen availability, Gln3 and Gat1 control the expression of SAP2 by regulating the Stp1 transcription factor (22). These studies suggested that Rhb1 and Tor1 kinase may also be related to SAP2 expression.

This study investigated the roles of Rhb1 and TOR signaling in C. albicans.

**MATERIALS AND METHODS**

**Strains and growth conditions.** Table 1 lists the C. albicans strains used in this study. All strains were routinely grown at 30°C in YPD medium (10 g/liter yeast extract, 20 g/liter peptone, 20 g/liter glucose) and synthetic defined (SD) medium (6.7 g/liter yeast nitrogen base without amino acids, 20 g/liter glucose, 0.79 g/liter complete supplement mixture [CSM] of amino acids; MP Biochemicals, Solon, OH). To determine cell growth and the expression levels of SAP2 and its gene product, a single colony from each strain was grown overnight at 30°C in YPD medium, washed, and subcultured at an optical density of 600 nm (OD600) of approximately 0.5 in YCB-BSA-YE (0.01% or 0.05% medium) (17). Gln3 and Gat1 control the expression of SAP2 by regulating the Stp1 transcription factor (22). These studies suggested that Rhb1 and Tor1 kinase may also be related to SAP2 expression.

This study investigated the roles of Rhb1 and TOR signaling in C. albicans.
-formed as described previously (72). For PCRs, 1 

acetic acid. Time courses were performed as described previously (72). For PCRs, 1 μl of cDNA served as the template for 1 cycle of 94°C for 10 min, followed by different numbers of cycles of 94°C for 30 s and 60°C for 30 s as indicated and 1 cycle of 72°C for 5 min. The PCR products were analyzed on 1.2% agarose gels and visualized by staining with SYBR Safe (Invitrogen, Carlsbad, CA).

Quantitative PCR was performed using MicroAMP optical 96-well reaction plates (catalog no. N801-0560; Applied Biosystems, Framingham, MA) and MicroAMP optical adhesive film (catalog no. 431197; Applied Biosystems), the SYBR green-based detection assay, and a 7500 real-time PCR system (Applied Biosystems). Each 20-μl reaction mixture consisted of 1 μl of cDNA, 0.3 μl of 20 μM of sense and antisense primers, 8.4 μl of water, and 10 μl of Power SYBR green PCR Master Mix (catalog no. 4367659; Applied Biosystems). The reaction conditions were 1 cycle of 50°C for 2 min and 95°C for 10 min, followed by 40 cycles of 95°C for 15 s and 60°C for 1 min. Each sample was amplified in triplicate, and each experiment was performed 3 times. Data analysis was performed with

TABLE 1. C. albicans strains used in this study

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SacI and KpnI digestion. The fragment was then transformed into 1 allele of the ADH1 locus of SC5314 to generate TetGFP.

RNA isolation, reverse transcription-PCR (RT-PCR), and real-time quantitative PCR. Total RNA isolation and cDNA synthesis were performed as described previously (72). For PCRs, 1 μl of cDNA served as the template for 1 cycle of 94°C for 10 min, followed by different numbers of cycles of 94°C for 30 s and 60°C for 30 s as indicated and 1 cycle of 72°C for 5 min. The PCR products were analyzed on 1.2% agarose gels and visualized by staining with SYBR Safe (Invitrogen, Carlsbad, CA).
Real-Time PCR System Sequence Detection software version 1.4 (Applied Biosystems). An average threshold cycle (Cₜ) value was obtained and normalized to the average Cₜ value of EF-1β. The comparative Cₜ method was used to quantify gene expression, and the relative expression was determined to be 2⁻ΔΔCₜ (36).

**Protein preparation and Western blotting.** Whole-protein lysate was prepared by suspending cells in 1% Nonidet P-40 (NP-40) extraction buffer (50 mM NaH₂PO₄ [pH 8.0], 150 mM NaCl, 1% NP-40) containing a 100-fold-diluted protease inhibitor cocktail (catalog no. P-8215; Sigma-Aldrich, St. Louis, MO) and 200 mM phenylmethylsulfonyl fluoride. Cells were broken using a vortex procedure with acid-washed glass beads for 30 s and immediately placed on ice for 30 s. This step was repeated 10 times. Soluble proteins were collected by centrifugation (13,300 × g for 10 min at 4°C) and quantified with a bicinchoninic acid (BCA) protein assay kit (Pierce, Rockford, IL). To detect secreted Sap2 and BSA in the medium, 20 μl of the supernatant was loaded onto a 12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) gel, and proteins were visualized with Coomassie blue staining. For Western blot analysis, proteins were subjected to 12% SDS-PAGE and transferred onto polyvinylidine difluoride membranes (Pall, Port Washington, NY) by the use of Towbin transfer buffer (24 mM Tris base, 192 mM glycine, 20% methanol) and a TE77 semi-dry transfer unit (GE Healthcare Bio-Sciences, Piscataway, NJ). Sap2 was detected with mouse monoclonal anti-Sap2 (catalog no. M166; Takara Bio, Shiga, Japan) (1:2,000 dilution). GFP was detected with JL-8 anti-GFP mouse monoclonal antibody (catalog no. 632380; Clontech, Palo Alto, CA) (1:2,000 dilution), and horseradish peroxidase (HRP)-conjugated goat anti-mouse IgG (Santa Cruz Biotechnology, Santa Cruz, CA) (1:10,000 dilution) was used as the secondary antibody. The chemiluminescence (ECL) was detected with Western Lightning Plus ECL reagents (Perkin-Elmer, Waltham, MA) and exposed to X-ray films (Fujifilm, Tokyo, Japan).

**One-hybrid assay.** A one-hybrid assay was performed as described previously (64). Briefly, cells were grown overnight in YPD broth at 30°C, washed, subcultured in fresh YCB–BSA–0.01% YE medium, and grown for 5.5 h to reach mid-log phase. The supernatant was removed by centrifugation (1,500 × g for 5.5 h) and the cell pellets were re-suspended in Z buffer (60 mM Na₂HPO₄, 40 mM NaH₂PO₄, 10 mM KCl, 1 mM MgSO₄, 7H₂O, and 4 μl/ml 98% β-mercaptoethanol; pH 7.0). Cells were then lysed by adding 15 μl of 0.1% SDS and 30 μl of chloroform and vortexing for 15 s. The suspensions were incubated for 30 min at 37°C in 0.2 ml of potassium phosphate buffer (pH 7.0) containing 4 mg of o-nitrophenyl-β-D-galactopyranoside/ml. The reactions were terminated with 0.4 ml of 1 M Na₂CO₃ when the suspension turned yellow. The supernatants from the reaction mixture were collected by centrifugation, and the optical density was measured at 420 and 550 nm. β-Galactosidase activity was calculated in Miller units as follows: 1 Miller unit = 1,000 × ([OD₄₂₀] − (1.75 × OD₅₅₀))/([OD₅₅₀] × T × V × OD₅₅₀), where T is the duration of the reaction (in minutes), V is the volume of the supernatant used in the assay (in milliliters), [OD₅₅₀] is the cell density at the beginning of the reaction, OD₅₅₀ is the absorbance derived from o-nitrophenyl-β-D-galactopyranosidase and light scattering from cell debris, and OD₅₅₀ tells the extent of absorption of light scattering from cell debris. Analysis was performed in triplicate based on 3 independent experiments.

**Cell susceptibility to rapamycin.** After growing overnight in YPD broth, cells were collected, washed, and suspended in sterile water. Ten-fold serial dilutions of the cultures were prepared at a concentration of approximately 2 × 10⁶ to 2 × 10⁸ cells/ml. Diluted cells (5 μl) were spotted onto YPD plates containing 0.01-μg/ml rapamycin (catalog no. 553210; Merck KGaA, Darmstadt, Germany) prepared as a 100 μg/ml stock by dissolution in methanol or YPD plates made with the same volume of methanol (as a control). Cell viability was recorded after incubation at 30°C for 2 days. This assay was performed independently 3 times.

**Analysis of Sap2 gene and protein levels.** The SAP2ex4A cell was incubated in fresh YCB–BSA–0.01% YE (0.01%) containing 30 mg/ml doxycycline to maintain the basal level of SAP2 expression. The cells were grown at 30°C for 6 h (to reach OD₆₀₀ = 2.5 to 3), and 5 ml of cell culture was then transferred into 50-ml conical tubes containing 0.2 mg/ml rapamycin (prepared from a stock of 100 μg/ml dissolved in 100% methanol) or an equal volume of methanol (0.2% [vehicle control]). The conical tubes were also filled with 1.8% CSM (MP Biochemicals) (prepared from a 20× stock) to determine the effects of amino acid availability. The cells were incubated at 30°C for different times and collected by centrifugation. The cell pellets and supernatants were used for RNA or protein isolation.

To correlate amino acid availability with intracellular Sap2 protein levels, SAP2ex4A cells were grown in YPD at 30°C overnight, washed, and subcultured in SD medium containing 30 mg/ml doxycycline at OD₆₀₀ = 0.5. The cells were grown at 30°C for 4 h (to reach OD₆₀₀ = 3.5 to 4.0), and 5 ml of cell culture treated with doxycycline was transferred into 50-ml conical tubes containing 0.2 mg/ml rapamycin or an equal volume of methanol (0.2% [vehicle control]). The cells were then incubated at 30°C for different times and collected by centrifugation. The resulting cell pellets were used for protein isolation.

**Analysis of GFP protein levels.** The TetGFP fusion strain carries a Ptet-GFP fusion that is similar to the construct in the SAP2ex4A strain, except the latter contains a Ptet-SAP2 fusion. The SAP2ex4A cell was incubated in fresh YCB–BSA–0.01% containing 30 mg/ml doxycycline. The growth conditions were the same as those described for analysis of Sap2 gene and protein levels. GFP protein levels were analyzed as described for protein preparation and detected by Western blotting.

**Proteolytic assay.** Cells were grown in YPD overnight and collected by centrifugation. The cell pellets were washed and resuspended in sterile water to approximately 5 × 10⁶ cells/ml. The resulting cell suspension (5 μl) was spotted onto YCB–BSA–0.01% plates (with 1.5% agar) containing 0.01 mg/ml rapamycin. The size of the clear zone surrounding the cells was measured after incubation at 37°C for 4 days.

**DNA microarray fabrication and sample preparation.** An Operon 70-mer probe set (Array-Ready Oligo Sets; AROS V1.2), containing 6,266 probes, and the C. albicans AROS upgrade set (V1.1), containing 1,659 probes, was used to construct DNA microarrays. These 70-mer oligonucleotide sets have been successfully used in studies on C. albicans and more than 10 different controls. These oligonucleotide sets have been successfully used in studies on C. albicans biol-
AM1753; Ambion). First-strand cDNA was prepared using total RNA and ArrayScript reverse transcriptase. After processing, DNA polymerase and RNase H were used for synthesis of the second-strand cDNA. To synthesize amino allyl UTP (aaUTP)-incorporated aRNA (amino allyl-modified aRNA), in vitro transcription was performed with T7 RNA polymerase and purified cDNA was used as a template. Cy dye coupling was performed after aRNA purification. Cy dye-coupled aRNA was purified by ultrafiltration using an RNAasy MinElute Cleanup kit (Qiagen). For biological replications, samples were prepared from 5 different batches of each wild-type and rhb1-deleted mutant cells. Dye swap experiments produced 5 sets of data for Cy3-labeled wild-type cells (Amersham Cy5 monoreactive dye pack [catalog no. PA25001]; GE Healthcare) and Cy3-labeled rhb1-deleted (CCT-D1) mutant cells (Amersham Cy5 monoreactive dye pack [catalog no. PA23001]; GE Healthcare). Another 5 sets were labeled by dye swapping (Cy3-labeled wild type versus Cy5-labeled rhb1-deleted mutant). Slides were prehybridized with 1% BSA, washed, and hybridized with Cy3- and Cy5-labeled aRNA in a Corning microarray hybridization chamber (catalog no. 2551; Corning). Hybridization was performed in a water bath at 65°C for 16 h. After hybridization, the slides were washed several times using SSC buffers with different stringencies. Slides were dried and scanned using an Axon GenePix 4000B scanner (Axon Instruments, Inc.) with wavelengths of 635 nm and 532 nm.

**DNA microarray data analysis.** The microarray data were first processed with LOWESS normalization to remove intensity-based variation across channels. The following linear model was then fitted to each spot hybridization chamber (catalog no. 2551; Corning). Hybridization was hybridized with Cy3- and Cy5-labeled aRNA in a Corning microarray labeled by dye swapping (Cy3-labeled wild type versus Cy5-labeled rhb1-deleted mutant). Slides were prehybridized with 1% BSA, washed, and hybridized with Cy3- and Cy5-labeled aRNA in a Corning microarray hybridization chamber (catalog no. 2551; Corning). Hybridization was performed in a water bath at 65°C for 16 h. After hybridization, the slides were washed several times using SSC buffers with different stringencies. Slides were dried and scanned using an Axon GenePix 4000B scanner (Axon Instruments, Inc.) with wavelengths of 635 nm and 532 nm.

**RESULTS**

**Rhb1 regulates cell growth in the presence of BSA by controlling Sap2 expression.** Sap2 is the major secreted protease of *C. albicans* in vitro (32, 81). Sap2 expression is induced in medium in which protein is the nitrogen source and is repressed in the presence of a more favorable nitrogen source, such as ammonium (22). Previous research showed that Rhb1 is involved in filamentation mediated by nitrogen starvation (72). This implies that Rhb1 may play a role in cell growth in medium in which protein is the major source of nitrogen. To test this hypothesis, *C. albicans* cells were grown in yeast carbon base (YCB) medium supplemented with bovine serum albumin (BSA) (as the nitrogen source) and a limited amount (0.01%) of yeast extract (YE) to reduce overall incubation time (22). Figure 1A depicts the growth rate of *C. albicans*. The rhb1-deleted strain showed a growth rate similar to those of the wild-type and RHBI-reconstituted strains. Cells in which RHBI expression was driven by the ADH1 promoter and RHBI was overexpressed (72) grew slower than wild-type cells. However, cells overexpressing RHBI exhibited an optical density similar to that of the wild type after prolonged incubation (Fig. 1A). The sap2-deleted mutant was used as a control, as it did not grow in YCB–BSA–YE (0.01%) medium (Fig. 1A) (21). The numbers of CFU were also counted (see Fig. S1 in the supplemental material) to avoid cell size or morphology variations that might have led to inaccurate ODsubeq{600} data for Fig. 1A. The pattern of cell growth derived from CFU counting was similar to that represented in Fig. 1A. However, the slow growth of the strain overexpressing RHBI might have been caused by other nitrogen sources in the medium. The concentration of yeast extract in the medium was increased to 0.05% to avoid this possibility. This diminished the slow growth of the strain overexpressing RHBI (see Fig. S2 in the supplemental material) and largely restored the defect of the sap2-deleted strain (see Fig. S2 in the supplemental material). These results indicate that the slow growth of the strain overexpressing RHBI and the growth defect of sap2-deleted strain are related to BSA utilization.

To further correlate cell growth with BSA utilization, supernatants of cells grown in YCB–BSA–YE (0.01%) were analyzed with SDS-PAGE. After 24 h of incubation, BSA was completely digested by the wild-type, rhb1-deleted, and RHBI-reconstituted strains (Fig. 1B). However, 2 independent strains overexpressing RHBI showed lower BSA utilization efficiency. The strains overexpressing RHBI degraded BSA slowly after 10 h of incubation compared to the wild type (Fig. 1B), and the molecular weight of degraded protein was relatively high. Even after 24 h of incubation, peptides or low-molecular-weight proteins derived from BSA degradation were still apparent in the strains overexpressing RHBI (Fig. 1B). The low efficiency of BSA degradation seen with RHBI overexpression confirms the low growth rate of the same strain (Fig. 1A).

The sap2-deleted mutant and a strain in which SAP2 is regulated by a tetracycline-inducible Prt1 promoter (65) were used as controls. The sap2-deleted mutant slightly degraded BSA, and the degradation was substantially slower (Fig. 1B). However, protein degradation became apparent in the SAP2-induced strain after the addition of doxycycline (a tetracycline antibiotic) (Fig. 1B). Western blotting using BSA antibody was performed to determine whether the lower-molecular-weight bands represented BSA or other proteins. The lower-molecular-weight bands were derived from BSA degradation (see Fig. S3 in the supplemental material). These results suggest that the protein utilization of the strain overexpressing RHBI is not as efficient as that of the other strains. Thus, the Rhb1 level may be correlated with the secretion or expression of secreted proteases.

Because Sap2 is the major secreted protease in vitro, the experiments in this study tested whether lower Sap2 expression levels result in delay of growth of the strain overexpressing RHBI. The expression of Sap2 was detected by Western blot analysis performed with Sap2 monoclonal antibody following previously described procedures (21). The rhb1-deleted mutant was the only mutant in which intracellular Sap2 was easily detected in cells after 12 h of incubation, as shown in Fig. 1C. The levels of extracellular Sap2 protein in other tested strains were too low to be detected. After 24 h of incubation, the expression levels of intracellular Sap2 seemed to reach saturation and were indistinguishable among the different samples. Nevertheless, the level of extracellular Sap2 in the strain overexpressing RHBI was much lower than in the others. The level of extracellular Sap2 in the rhb1-deleted strain was slightly higher than in the wild-type and RHBI-reconstituted...
strain. After prolonged incubation (48 h), the level of the protein remaining in the medium was relatively low. Thus, Sap2 might not be required and was proteolyzed, further reducing the intracellular level of Sap2 compared to that seen in cells after 24 h of incubation. The different extracellular Sap2 levels among the strains were still apparent after 12 h but were almost indistinguishable after 24 h of incubation. Although the levels of Sap2 protein were higher in the \textit{rhb1}-deleted mutant than in the wild type (Fig. 1C), the doubling time for the strain overexpressing \textit{RHB1} in YCB–BSA–YE (0.01%) was approximately 6.1 ± 0.02 h. (B) BSA degradation by \textit{C. albicans} after 10 and 24 h of incubation. Each cell supernatant (20 μl) was analyzed by 12% SDS-PAGE, and proteins were visualized by Coomassie blue staining. Data represent the results of only 1 experiment, although experiments were performed at least 3 times with identical results. The rectangular box indicates peptides or low-molecular-weight proteins derived from BSA degradation. The arrow indicates the intact BSA (66 kDa). M, protein molecular marker; MW, molecular weight. (C) Expression of extracellular and intracellular Sap2 in different \textit{C. albicans} strains. Cells were grown as described in panel A for 12, 24, and 48 h, and Sap2 was detected by Western blotting. The nonspecific band served as the loading control and was used to normalize the intracellular Sap2 levels indicated by the fold change values. (D) Comparison of levels of cell growth on YPD and YCB–BSA–YE (0.01%) agar plates. Cells were grown overnight in YPD, washed, serially diluted 10-fold, and spotted onto agar plates. Plates were photographed after incubation at 30°C for 1 day. Data are representative of at least 3 independent experiments with identical results. Cell numbers are shown at the top of the panels.

FIG 1 Rhb1 regulates cell growth in medium with BSA as the major nitrogen source by controlling \textit{C. albicans} Sap2 expression. (A) Cells were grown in YPD broth overnight, washed twice with sterile water, and subcultured in YCB–BSA–YE (0.01%) medium (initial cell density, OD\(_{600}\) = 0.5) at 30°C. The \textit{C. albicans} strains used in this experiment were SC5314 (wild type; filled triangles), CCT-D1 (\textit{rhb1} deleted; open squares), CCT-RD1 (\textit{RHB1} reconstituted; open circles), CCT-OE1 (overexpressing \textit{RHB1}; filled circles), and SAP2MS4A (\textit{sap2} deleted; filled squares). The doubling times for wild-type, \textit{rhb1}-deleted, and \textit{RHB1}-reconstituted strains were approximately 5.7 ± 0.2 h, 5.8 ± 0.2 h, and 5.7 ± 0.1 h, respectively. The doubling time for the strain overexpressing \textit{RHB1} in YCB–BSA–YE (0.01%) was approximately 6.1 ± 0.4 h. (B) BSA degradation by \textit{C. albicans} after 10 and 24 h of incubation. Each cell supernatant (20 μl) was analyzed by 12% SDS-PAGE, and proteins were visualized by Coomassie blue staining. Data represent the results of only 1 experiment, although experiments were performed at least 3 times with identical results. The rectangular box indicates peptides or low-molecular-weight proteins derived from BSA degradation. The arrow indicates the intact BSA (66 kDa). M, protein molecular marker; MW, molecular weight. (C) Expression of extracellular and intracellular Sap2 in different \textit{C. albicans} strains. Cells were grown as described in panel A for 12, 24, and 48 h, and Sap2 was detected by Western blotting. The nonspecific band served as the loading control and was used to normalize the intracellular Sap2 levels indicated by the fold change values. (D) Comparison of levels of cell growth on YPD and YCB–BSA–YE (0.01%) agar plates. Cells were grown overnight in YPD, washed, serially diluted 10-fold, and spotted onto agar plates. Plates were photographed after incubation at 30°C for 1 day. Data are representative of at least 3 independent experiments with identical results. Cell numbers are shown at the top of the panels.
the wild-type and rhl1-deleted strains showed similar growth rates in YCB–BSA–YE (0.01%) liquid medium (Fig. 1A). A possible explanation is that the rhl1-deleted mutant may exhibit a growth delay phenotype when growing in a nutrient-rich environment. To test this possibility, cells were grown in YPD-rich medium and compared to cells grown in a medium in which protein is the major nitrogen source. Equal numbers of cells were serially diluted and spotted onto YPD and YCB–BSA–YE (0.01%) agar plates. The rhl1-deleted mutant showed a slight delay in cell growth on YPD but not on YCB–BSA–YE (0.01%) agar plates (Fig. 1D). This suggests that high levels of Sap2 protein expression (Fig. 1B and C) may somehow compensate for the growth delay of the rhl1-deleted mutant in YCB–BSA–YE (0.01%) medium (Fig. 1A and D). The spot assay somehow masked the minor growth delay of strain overexpressing RHB1 shown in the liquid medium (Fig. 1A). It is possible that the spot assay is not sensitive enough to detect a minor delay in cell growth. Overall, these results indicate that Rhl1 is involved in the expression of Sap2, which is critical for cell growth in media with protein as the major nitrogen source.

The TOR signaling pathway is also involved in regulating Sap2 expression. Rapamycin is an inhibitor of Tor kinase and forms a complex with FKBP12, a 12-kDa protein (28). S. cerevisiae cells treated with rapamycin express a phenotype that mimics that of cells undergoing nitrogen starvation (14). Previous research showed that mutants lacking Rhl1 are hypersensitive to rapamycin, suggesting that Rhl1 plays a role in TOR signaling (72). Figure 2A illustrates the close relationship among Rhl1, rapamycin, and Tor1. The rhl1-deleted mutant was rapamycin sensitive whereas the TOR1-TOR1 mutant was rapamycin resistant compared with the wild type (Fig. 2A). The C. albicans TOR1-1/TOR1 strain carries a TOR1-1 allele with a point mutation (TOR1S1984I) in the FKBP12 rapamycin-binding (FRB) domain of the Tor1 kinase (20). The TOR1-1/TOR1 strain is resistant to rapamycin, because the FKBP12-rapamycin complex cannot bind to Tor1 kinase with this mutation (20). Two independent constructs of the rhl1 deletion on the TOR1S1984I mutant background were not as strongly resistant to rapamycin as TOR1-1/TOR1 (Fig. 2A). In fact, rhl1 deletion in the TOR1-1/TOR1 background showed slightly enhanced resistance to rapamycin compared with the rhl1-deleted mutant. These results suggest that deletion of RHB1 compensates for the defect in rapamycin binding to the mutant FRB domain of the Tor1 kinase. Rapamycin and the rhl1 deletion combined have an additional effect on the Tor1 kinase, thus reinforcing rapamycin susceptibility.

The level of Sap2 is altered in the rhl1-deleted strain and the strain overexpressing RHB1 when protein is the major nitrogen source (Fig. 1B and C). Thus, it is possible that both Rhl1 and Tor1 kinase are involved in regulating SAP2 gene expression. Total RNA was isolated and analyzed by reverse transcription-PCR (RT-PCR). After 10 h of growth in YCB–BSA–YE (0.01%) medium, the expression level of the SAP2 transcript was slightly higher in the rhl1-deleted strain than in the wild-type and RHB1-reconstituted strains (Fig. 2B). However, the expression of SAP2 transcripts was nearly undetectable in the strain overexpressing RHB1 after 10 h (Fig. 2B). Expression of SAP2 reached similar levels in all strains tested after 24 h. To determine the role of Tor1 kinase in SAP2 expression, the experiments in this study used synthetic defined (SD) medium, which contains no protein but has ammonium, a favorable nitrogen source, and a complete supplement mixture (CSM) of amino acids. The cultures were also treated with and without rapamycin. As expected, rapamycin-induced SAP2 expression at a level greater than that seen with vehicle-treated control cells (Fig. 2C). The extracellular proteolytic activity of Sap2 was also assessed (38) by spotting the cells onto YCB–BSA–YE (0.01%) agar plates containing rapamycin. A high concentration of cells (~2.5 × 10⁶ cells/spot) was used for each tested strain to compensate for the possible reduction in cell growth caused by rapamycin. The zones of proteolysis surrounding the cell spots represent the relative levels of protease secretion (38). The wild-type strain produced a larger proteolytic zone than the TOR1-1/TOR1 strain (Fig. 2D). The sap2-deleted strain served as a negative control and did not show a clear proteolytic zone (Fig. 2D). These results indicate that Tor1 kinase is involved in SAP2 expression and somehow affects the secretion of Sap2.

To further correlate the TOR signaling pathway with SAP2 expression, transcription factors that may be downstream of the Tor1 kinase were also examined during rapamycin treatment. In S. cerevisiae, Gln3 and Gat1 are the downstream effectors of TOR signaling in regulating nitrogen catabolism (7). In C. albicans, Gln3 and Gat1 activate the Stp1 transcription factor, which in turn controls SAP2 expression (22). A time course analysis of SAP2 expression was performed using RT-PCR. SAP2 was activated with rapamycin treatment in wild-type cells, whereas a relatively low level of SAP2 was expressed in the TOR1-1/TOR1 strain (Fig. 3A). After rapamycin treatment, cells lacking Gln3 or Gat1 showed a delay and a decrease in SAP2 expression compared with wild-type cells. Moreover, the gln3Δ/gat1Δ double mutant showed an even lower level of SAP2 expression than the gln3Δ and gat1Δ single mutant strains (Fig. 3A). With rapamycin treatment, deletion of STP1 also decreased SAP2 expression (Fig. 3A). The sap2-deleted strain served as a negative control. This suggests that SAP2 expression controlled by TOR signaling involves Gln3 or Gat1 or both and is further activated by Stp1. To further determine the function of Stp1 in TOR signaling, this study examined the susceptibility of the stp1-deleted mutants to rapamycin. As with the gln3Δ and gat1Δ-deleted mutants, the stp1Δ-deleted mutants were resistant to rapamycin (Fig. 3B) (40). The S. cerevisiae stp1Δ-deleted mutant is rapamycin sensitive (66), suggesting that the Stp1 proteins in the two budding yeasts may function differently.

The transcriptional activity of Gat1 and Stp1 was assessed with a one-hybrid analysis (64) using cells grown in YCB–BSA–YE (0.01%) medium (Fig. 3C). LexA-Gcn4 and LexA constructs served as positive and negative controls, respectively (31). Compared to wild-type cells, Gat1 and Stp1 showed relatively higher transcriptional activity in cells on the rhl1-deleted background. However, the transcriptional activity of Gat1 (but not Stp1) was substantially lower than that seen with the wild type in the RHB1 overexpression background (Fig. 3C). These results suggest that Rhl1 and Tor1 are involved in the activity of Gln3 and Gat1 in transcriptional regulation.

Tor1 and amino acid regulation of Sap2 protein level. Because Tor1 is involved in SAP2 transcription and somehow affects Sap2 secretion (Fig. 2B and C), we hypothesized that Tor1 may affect Sap2 directly at the protein level. This possibility was tested using a strain in which SAP2 is regulated by the Ppet promoter (Fig. 1B). In this strain, SAP2 expression is likely to be activated by doxycycline independently of TOR signaling. When doxycycline was added, cells showed almost equal levels of SAP2 transcription throughout all examined time points, in both the presence and absence of rapamycin (Fig. 4A). How-
ever, the levels of both extracellular and intracellular Sap2 in the presence of rapamycin were higher than those in control cells without rapamycin treatment (Fig. 4B). A strain carrying a \textit{P} \textit{tet}-GFP fusion was also tested to rule out the possibility that the rapamycin-related effect on the level of Sap2 protein was due to the construction of the \textit{P} \textit{tet} promoter. GFP was expressed at similar levels under the same growth conditions (see Fig. S4 in the supplemental material). The data suggest that the rapamycin-related effect on the level of Sap2 protein was due to the construction of the \textit{P}\textit{tet} promoter. GFP was expressed at similar levels under the same growth conditions (see Fig. S4 in the supplemental material). The data suggest that

\textbf{FIG 2} Tor1 kinase regulates \textit{SAP2} expression and affects Sap2 protease secretion. (A) Epistatic interaction between Rhb1 and Tor1 kinase. Tenfold serial dilutions of each strain were prepared and spotted onto YPD with 0.01-μg/ml rapamycin or with 0.1% methanol (MeOH [the vehicle control]). Plates were incubated at 30°C for 2 days. Data are representative of at least 3 independent experiments with identical results. Cell numbers are shown at the top of the panels. (B) \textit{SAP2} expression in different \textit{C. albicans} strains. Cells were grown as described in the Fig. 1A legend for 10 and 24 h, and \textit{SAP2} expression was analyzed with RT-PCR. \textit{EFB1} served as an endogenous control. Cn, number of cycles in the PCRs. The semiquantitative levels of \textit{SAP2} induction were calculated and plotted. (C) Induction of \textit{SAP2} expression by rapamycin treatment. The SC5314 strain was incubated in SD medium at 30°C until reaching mid-log phase. The cell pellets were washed and subsequently subcultured in SD (−), SD containing 0.2% methanol (MeOH [the vehicle control]), or SD containing 0.2-μg/ml rapamycin. After cells were grown for the indicated times, the level of \textit{SAP2} expression was detected with RT-PCR. \textit{EFB1} served as an endogenous control. The semiquantitative levels of \textit{SAP2} induction were calculated and plotted. m, minutes. (D) Assay for Sap2 secretion by BSA degradation. Approximately 2.5 × 10^5 cells of the SC5314, JRB12 (TOR1-1/TOR1), and SAP2MS4A (sap2Δ/sap2Δ) strains were spotted on YCB–BSA–YE (0.01%) agar plates containing rapamycin (0.01 μg/ml) and incubated at 37°C for 4 days. The double arrows indicate the diameter of the proteolytic zone.

However, the levels of both extracellular and intracellular Sap2 in the presence of rapamycin were higher than those in control cells without rapamycin treatment (Fig. 4B). A strain carrying a \textit{P}\textit{tet}-GFP fusion was also tested to rule out the possibility that
Gln3, Gat1, and Stp1 regulate SAP2 expression downstream of the Tor1 kinase. (A) The gln3-,-gat1-,- and stp1-deleted strains attenuate rapamycin-induced SAP2 expression. Cells were grown in YPD medium at 30°C until reaching the mid-log phase. The cell pellets were washed and subcultured in YPD with and without 0.2 μg/ml of rapamycin. After cells were grown for the indicated times, the level of SAP2 expression was detected with RT-PCR. The strains used in this experiment include SC5314 (wild type), JRB12 (TOR1-1/TOR1), CCT-D1 (rhb1Δ/rhb1Δ), STP1M4A (stp1Δ/stp1Δ), GLN3M4A (gln3Δ/gln3Δ), GAT1M4A (gat1Δ/gat1Δ), Δgln3GAT1M4A (gln3Δ/gln3Δ gat1Δ/gat1Δ), and SAP2MS4A (sap2Δ/sap2Δ). EFB1 served as an endogenous control. The semiquantitative levels of SAP2 induction were calculated and plotted. The sample of wild type (without rapamycin treatment) from time zero was used as the calibrator. m, minutes. (B) Stp1 is involved in the TOR signaling pathway. SC5314, JRB12 (TOR1-1/TOR1), STP1M4A, and CHIA-D1 (the last 2 are independent stp1Δ/stp1Δ mutants as shown in the third and fourth lanes from the top, respectively) were incubated in YPD overnight. The cell pellets were washed, serially diluted, spotted onto YPD agar plates with 0.01 μg/ml rapamycin or 0.1% methanol (MeOH [the vehicle control]), and incubated at 30°C for 2 days. Data are representative of 3 independent experiments with identical results. Cell numbers are shown at the top of the panels. (C) Yeast one-hybrid analysis demonstrated that Rhb1 affects the transcriptional activity of Gat1 and Stp1. Strains with and without the LexA operator upstream of the basal promoter of the lacZ reporter gene are labeled LexA operator (CCR1 background) and basal (COP1 background), respectively. COP1 and CCR1 were constructed in the rhb1-deleted (rhb1Δ/rhb1Δ) or RHB1-overexpressing (PADH1::RHB1) background. Cells were incubated in YCB–BSA–YE (0.01%) medium at 30°C for 5.5 h, and the LacZ activity modulated by Stp1-LexA or LexA-Gat1 binding to the LexA operator was measured with a liquid β-galactosidase assay. β-Galactosidase data are shown in Miller units and represent averages of the results of 3 independent experiments. *, P ≤ 0.05; **, P ≤ 0.01; ***, P ≤ 0.005. The error bars show standard deviations.
TOR signaling is involved in regulating SAP2 transcription and further affects the expression of Sap2 protein.

This discussion and previous research (22) indicate that nitrogen depletion and the presence of protein function as signals for SAP2 activation in C. albicans through Rhb1-TOR signaling. This study further determined whether the Sap2 protein level is also affected by the availability of amino acids, another possible source of nitrogen. We detected Sap2 in response to treatment with amino acids and rapamycin (Fig. 5). After 3.5 h of incubation with doxycycline, the intracellular levels of Sap2 were significantly abated in the strain in which SAP2 was regulated by the P tet promoter when a combination of amino acids (CSM) was added to the nitrogen-poor YCB-BSA-YE (0.01%) medium (Fig. 5A). The reduction of Sap2 protein levels by a high concentration of amino acids did not occur in the presence of rapamycin (Fig. 5A). In the amino acid-containing SD medium, Sap2 was not detected even when SAP2 transcription was turned on with doxycycline (Fig. 5B). In contrast, Sap2 was first detected after cells were treated with rapamycin for 1 h (Fig. 5B). These results indicate that amino acid starvation is also a signal for Sap2 protein expression through TOR signaling.

Identification of Rhb1-regulated genes in C. albicans. This study also investigated possible target genes of C. albicans Rhb1 with DNA microarray analysis. Both wild-type and rhb1-deleted strains were grown in SD medium to mid-log phase, and total RNA was isolated. Samples were prepared and labeled with Cy3 or Cy5 fluorescent dye before data analysis. Using a ρ value of ≤0.0148 as the cutoff, expression of 109, 17, and 6 gene transcripts changed at least 1.5-, 2-, and 3-fold, respectively, in the rhb1-deleted mutant compared with levels in the wild type (Fig. 6A). The following discussion reviews the highlights of the 113 genes, and the details are listed in Tables S1 and S2 in the supplemental material. In addition to SAP2, the expression of genes such as MEP2, GAP2, RBT1, OPT1, and ALS3 was upregulated in the rhb1 mutant. MEP2 encodes an ammonium sensor and transporter (10), and its regulation by Rhb1 is consistent with previous findings (72). The GAP2 and OPT1 gene products are a general amino acid permease and an oligopeptide transporter, respectively (47). The RBT1 gene product is predicted to be a cell wall protein (6). ALS3 encodes an adhesin that plays a complementary role in C. albicans biofilm formation (52, 53). Tor1 kinase also regulates RBT1 and ALS3 (6), and GAP1, HIP1, and EFG1 are among the downregulated genes in the rhb1-deleted mutant. GAP1 encodes a general amino acid permease, and EFG1 encodes a transcription factor controlling C. albicans morphogenesis (52, 53). HIP1 encodes an uncharacterized protein regulated by Gcn2 and Gcn4 (70), and its sequence shows the best hit with S. cerevisiae GAP1. Real-time quantitative PCR verified the expression patterns of GAP1, GAP2, and HIP1 (Fig. 6B).

DISCUSSION

The Ras superfamily of small GTPases is generally classified into 5 families, including Ras, Rho, Rab, Arf, and Ran (3). Of the Ras members, Ras homolog enriched in brain (Rheb) is a novel and unique small G protein (3). Most of the information on Rheb has been gathered from mammal studies. Transcriptionally controlled tumor protein (TCTP; also known as p23) and tuberous sclerosis complex 2 (Tsc2) act as a guanine nucleotide exchange factor (GEF) and a GTPase-activating protein (GAP), respectively, to
regulate Rhb1 (25, 57). Rhee is likely a positive regulator of the mTOR kinase and activates mTORC1 signaling (42).

The functions of Rbh1 (the homolog of Rhee) in fungi are not well characterized. In S. cerevisiae, Rbh1 functions in the uptake of arginine (75). However, no homolog of mammalian Tsc2 has been found in the genome of S. cerevisiae (3). Whether environmental signals can be conveyed from Rbh1 to TORC1 remains unknown. Rbh1 regulates amino acid uptake, mating, cell growth, cell cycle progression, and stress response in S. pombe (44, 73, 74, 76). Mutations in TSC2 cause defects in the uptake of arginine and leucine (77, 80) and lead to a delayed response in nitrogen starvation-mediated G1 arrest (48, 78). In fission yeast, TORC1 controls cell proliferation in response to nutrient signals and acts downstream of Rbh1 (51, 76).

Previous research identified Tsc2 and Rbh1 in C. albicans and linked Rbh1 to the Tor1 kinase (72). Rbh1 and Tor1 are involved in nitrogen starvation-induced morphogenesis by controlling MEP2 and cell wall integrity (6, 72). The results of the current study also indicate that the Rbh1-TOR signaling pathway controls the expression of the C. albicans virulence factor Sap2. In medium containing protein as the major nitrogen source, RHH1 overexpression attenuates the expression of Sap2 before 24 h but is otherwise normal (Fig. 1C). In the absence of RHH1, cells express Sap2 before other strains but otherwise function as normal (Fig. 1C). Although intrinsic growth defects appeared in the rhh1-deleted strain (Fig. 1D), a higher level of Sap2 may somehow compensate for this growth defect and result in a similar growth rate in the YCB–BSA–YE (0.01%) medium (Fig. 1A).

Rapamycin treatment also activated Sap2 in nitrogen-rich and no-protein conditions, which are generally unfavorable for Sap2 transcription (Fig. 2B). Rapamycin also enhanced the expression of Sap2 (Fig. 2B). The increased expression of Sap2 and its gene product somehow produced highly efficient Sap2 secretion (Fig. 1C and 2C). This result agrees with previous research showing that Sap synthesis and secretion are tightly coupled (30). Although the mechanisms of this process remain unclear, almost all S. cerevisiae mutants lacking vacuolar protein sorting (VPS) genes show susceptibility to rapamycin (82). Researchers recently reported that C. albicans mutants lacking VPS1 and VPS4 were defective in Sap2 secretion in an extracellular environment (8, 38). Moreover, we also tested a vps4-deleted strain and a strain in which VPS1 was repressed by doxycycline and found that both were hypersensitive to rapamycin (data not shown). The mechanisms of VPS-mediated Sap2 secretion and Rbh1-TOR signaling are under investigation in our laboratory.

The transcription factors Gln3 and Gat1 regulate another transcriptional factor, Stp1, which in turn controls Sap2 (22). This report links the Rbh1-TOR signaling pathway with this Stp1-mediated Sap2 regulation (Fig. 3A and B). The Csy1, Ptp3, and Ssy5 (S5) sensor complex likely regulates the activities of C. albicans Stp1 and another related transcriptional factor, Stp2 (47). In the presence of amino acids, Stp1 and Stp2 undergo proteolytic cleavage by the activated SPS complex. Under these conditions, processed Stp1 and Stp2 lack N-terminal inhibitory domains and are translocated from the cytosol to the nucleus. Processed Stp1 regulates protein utilization genes such as SAP2 and OPT1, whereas processed Stp2 regulates genes related to amino acid utilization, including the amino acid permeases Gap1 and Gap2 (47). Expression of some other genes (e.g., an oligopeptide transporter [OPT3]) can be induced by either processed Stp1 or Stp2. DNA microarray analysis revealed that OPT1, GAP2, and SAP2 were upregulated after deletion of RHH1, whereas GAP1 and HIP1 were downregulated (see Tables S1 and S2 in the supplemental material). These permeases may be responsible for assimilating extra-
### Figure 6

Identification of Rhb1 target genes by the use of DNA microarray analysis. 

(A) Summary of the fold change thresholds of the differentially expressed genes in the *rhl1*-deleted mutant (*CCT*-D1) compared with the wild type (*SC5314*). RNAs were collected after both strains were grown to mid-log phase in SD medium at 30°C. 

(B) Relative expression levels of three general amino acid permease genes: *GAP1*, *HIP1*, and *GAP2*. Quantitative real-time PCR was performed for *GAP1*, *HIP1*, and *GAP2* in the wild-type (*SC5314*), *CCT*-D1 (*rhl1Δ/rhl1Δ*), and *CCT*-RD1 (*RHB1*-reconstituted) strains. Cells were grown in SD medium for 4 h to mid-log phase under the same conditions used in the microarray experiment. The threshold cycle (*CT*) value of each gene was derived from the average of the results of 3 experiments. The *ΔCT* value was determined by subtracting the average *ΔCT* of endogenous control *EFB1* from the average *CT* of the gene tested. The *ΔΔCT* of each gene was calculated by subtracting the *ΔCT* value of the corresponding calibration value (wild-type sample). The average *ΔΔCT* and standard deviation values were determined from experiments performed in triplicate. The relative expression level of each gene is 2^-ΔΔCT, ***, P ≤ 0.005. The error bars show the standard deviation.
Sap2 protein level by TOR signaling is part of the cellular response to amino acid availability. Starvation resulting from amino acid deficiency activates the GAAC pathway in *S. cerevisiae* (29), and the GAAC is integrated with the TOR signaling pathway (68). The GAAC pathway is poorly understood in *C. albicans*. Although Gcn4 regulates the expression of amino acid synthetic genes in response to amino acid starvation, regulation of Gcn4 mRNA does not seem to occur (71). The *C. albicans* ortholog of Gcn2 also has limited involvement in the cellular response to amino acid starvation. This is likely because Gcn2 inactivation only partially attenuates cell growth under conditions of amino acid starvation (70). However, the regulation of amino acid biosynthetic genes is still dependent on Gcn4 (70). The DNA microarray analysis in this study confirms that several amino acid biosynthetic and Gcn4 target genes were differentially expressed in the *rhb1*-deleted mutant (see Tables S1 and S2 in the supplemental material). This suggests that GAAC regulation in *C. albicans* may occur through the Rhb1-TOR signaling pathway. Future research on this topic will investigate the details of the *C. albicans* GCN-like regulation mediated by Rhb1 and TOR signaling.

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REFERENCES

11. Brand A, MacCallum DM, Brown AJ, Gow NA, Odds FC. 2004. Tor-regulated expression of *ura3* in response to amino acid availability. Starvation resulting from amino acid deficiency activates the GAAC pathway in *S. cerevisiae* (29), and the GAAC is integrated with the TOR signaling pathway (68). The GAAC pathway is poorly understood in *C. albicans*. Although Gcn4 regulates the expression of amino acid synthetic genes in response to amino acid starvation, regulation of Gcn4 mRNA does not seem to occur (71). The *C. albicans* ortholog of Gcn2 also has limited involvement in the cellular response to amino acid starvation. This is likely because Gcn2 inactivation only partially attenuates cell growth under conditions of amino acid starvation (70). However, the regulation of amino acid biosynthetic genes is still dependent on Gcn4 (70). The DNA microarray analysis in this study confirms that several amino acid biosynthetic and Gcn4 target genes were differentially expressed in the *rhb1*-deleted mutant (see Tables S1 and S2 in the supplemental material). This suggests that GAAC regulation in *C. albicans* may occur through the Rhb1-TOR signaling pathway. Future research on this topic will investigate the details of the *C. albicans* GCN-like regulation mediated by Rhb1 and TOR signaling.

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