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Mds3 Regulates Morphogenesis in *Candida albicans* through the TOR Pathway▽†

Lucia F. Zacchi, Jonatan Gomez-Raja, and Dana A. Davis*

Department of Microbiology, University of Minnesota, Minneapolis, Minnesota 55455

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The success of *Candida albicans* as a major human fungal pathogen is dependent on its ability to colonize and survive as a commensal on diverse mucosal surfaces. One trait required for survival and virulence in the host is the morphogenetic yeast-to-hypha transition. Mds3 was identified as a regulator of pH-dependent morphogenesis that functions in parallel with the classic Rim101 pH-sensing pathway. Microarray analyses revealed that *mds3*Δ/Δ cells had an expression profile indicative of a hyperactive TOR pathway, including the preferential expression of genes encoding ribosomal proteins and a decreased expression of genes involved in nitrogen source utilization. The transcriptional and morphological defects of the *mds3*Δ/Δ mutant were rescued by rapamycin, an inhibitor of TOR, and this rescue was lost in strains carrying the rapamycin-resistant *TOR1-1* allele or an *rbp1*Δ/Δ deletion. Rapamycin also rescued the transcriptional and morphological defects associated with the loss of Sit4, a TOR pathway effector, but not the loss of Rim101 or Ras1. The *sit4*Δ/Δ and *mds3*Δ/Δ mutants had additional phenotypic similarities, suggesting that Sit4 and Mds3 function similarly in the TOR pathway. Finally, we found that Mds3 and Sit4 coimmunoprecipitate. Thus, Mds3 is a new member of the TOR pathway that contributes to morphogenesis in *C. albicans* as a regulator of this key morphogenetic pathway.

Eukaryotic cell growth and morphogenesis is affected by numerous environmental signals. These environmental signals are integrated by highly conserved regulators, including Ras, protein kinase C (PKC), and the target of rapamycin (TOR). These regulators govern the activity of signal transduction pathways, which generally promote changes in gene expression affecting an appropriate cellular response to the environmental signal. TOR is an essential kinase conserved throughout eukaryotic evolution. In mammalian systems, mTOR is required for embryogenesis (38) and for cellular morphogenesis in neurons (33), vacuolar smooth-muscle cells (53), and T cells (19). In the model yeast *Saccharomyces cerevisiae*, TOR plays a fundamental role in morphogenesis, being required for pseudohyphal growth and sporulation (14, 80). TOR governs morphogenesis by regulating numerous biological processes, including autophagy, translation, and ribosome biogenesis (79). Thus, in eukaryotic cells, TOR responds to environmental signals to promote growth and morphogenetic changes.

In the fungal opportunistic pathogen *Candida albicans*, the most well-studied morphogenetic transition is the yeast-hypha switch. The switch between the yeast and hyphal growth forms plays a critical role in the ability of *C. albicans* to colonize as a commensal and cause disease as a pathogen. Yeast cells are small, are nonadherent, and are less immunogenic and divide more rapidly than hyphal cells, which may allow *C. albicans* to successfully outcompete the faster-growing bacterial flora and the immune responses as well as pass through capillary beds during disseminated disease. Hyphal cells are long and extremely adherent, promote profound immune responses, and divide more slowly. Further, hyphal cells secrete numerous degradative enzymes and are invasive (32, 56). Hyphal cells may allow *C. albicans* to maintain itself on mucosal surfaces and, in immunocompromised hosts, enter the bloodstream. Both yeast and hyphal cells are observed in commensally colonized and diseased sites, demonstrating the relevance of this morphogenetic switch for *C. albicans* survival in the host. This idea is further supported by genetic analyses demonstrating that *C. albicans* mutants locked in a yeast or filamentous form are unable to cause disease (48, 58). Thus, the yeast-hypha morphogenetic switch is critical for pathogenesis.

Because the yeast-hypha transition is critical for pathogenicity, much work has been devoted to identifying the environmental sensors and signal transduction pathways that respond to these signals. The yeast-hypha morphogenetic switch is governed by a plethora of environmental cues, including pH, nutrient availability, temperature, and host factors (3, 10, 17, 56). For example, neutral-alkaline environmental pH is sensed by the Rim101 signal transduction pathway, resulting in the proteolytic activation of the Rim101 transcription factor, which promotes hyphal formation (16, 62, 63). Loss-of-function mutants in the Rim101 pathway do not form hyphae in response to neutral-alkaline environmental pH and show reduced virulence in animal models of infection (15, 61). Similarly, nutrient levels are sensed by the TOR pathway, which also promotes hyphal formation and is required for pathogenesis (4, 14, 46). These studies highlight the profound link between morphogenesis and virulence in the host in response to environmental signals, such as pH and starvation.

To gain further insights into *C. albicans* adaptation to envi-

* Corresponding author. Mailing address: 1360 Mayo Building MMC196, University of Minnesota, 420 Delaware St. SE, Minneapolis, MN 55455. Phone: (612) 624-1912. Fax: (612) 626-0623. E-mail: dadavis@umn.edu.

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ronmental pH, we identified additional signal transduction pathways that contribute to adaptation to environmental pH. For example, calcineurin and its associated transcription factor, Crz1, were found to act in parallel with Rim101 for adaptation to alkaline pH in *C. albicans* (43). Further, using a forward genetics approach, *MDS3* was identified as a positive regulator of alkaline pH responses in *C. albicans* (18). Like calcineurin, Mds3 was shown to act in parallel to Rim101 to promote neutral-alkaline pH responses. Mds3 also governs adaptation to neutral-alkaline pH responses in *S. cerevisiae*; however, this function originally was masked by the presence of the redundant Mds3 paralog, Pmd1. While the well-studied Rim101 pathway is required for adaptation to environmental pH, Mds3 plays a key role in adaptation to environmental pH.

MDS3 originally was identified in *S. cerevisiae* as a positive regulator of meiosis, a morphogenetic process that requires an alkaline environment (5, 24). In *C. albicans*, Mds3 is involved in a variety of morphogenetic processes, including the yeast-hyphal transition, chlamydospore formation, and biofilm formation (18, 60, 65). Despite a clear role for Mds3 in diverse morphogenetic processes in fungi, how Mds3 promotes morphogenesis is unknown. Work done in *S. cerevisiae* suggested that Mds3 transmits starvation signals to Ras (5, 54). However, we demonstrate that Mds3 contributes to morphogenesis in *C. albicans* through the negative regulation of the TOR pathway. We find that an *mds3*Δ/Δ mutant has transcriptional defects indicative of a hyperactive TOR pathway and that these transcriptional defects are rescued by rapamycin, a TOR pathway inhibitor. Further, the filamentation defects associated with the *mds3*Δ/Δ mutant are rescued by rapamycin. We also demonstrate that the loss of Sit4, a downstream effector of TOR, mimics the loss of Mds3. In *C. albicans*, the loss of Sit4 or Mds3 results in resistance to rapamycin, whereas in *S. cerevisiae* this loss results in sensitivity to rapamycin, suggesting that TOR functions are distinct in these two species. Finally, we demonstrate that Mds3 and Sit4 interact, suggesting a mechanism of TOR pathway regulation. In total, our results establish that Mds3 is a member of the TOR pathway and suggest that Mds3 regulates *C. albicans* development and morphogenesis through the TOR pathway.

MATERIALS AND METHODS

***C. albicans* strains and plasmids.** All yeast strains used in this study are listed in Table 1. DAY938 was constructed by deleting the second copy of *MDS3* from VIC1, the *MDS3*/*mds3*::*ARG4* parent of strain VIC3 (18), using an *mds3*::*URA3-dpl200* disruption cassette amplified with primers MDS3 5DR and MDS3 3DR (Table 2). Strains DAY1122 and DAY1123 were constructed by transforming strains DAY938 and DAY286, respectively, with the *tor1*::*HIS1* disruption cassette, which was amplified using primers TOR1 5DR and TOR1 3DR (Table 2). All deletions are from the start to the stop codon and were generated by chemical transformation (78). The correct integration of the disruption cassettes was verified by PCR using the Mds3null 5-detect and Mds3null 3-detect primers or TOR1 5′detect-2 and TOR1 3′detect primers (Table 2). DAY1118 and DAY1119 were generated by transforming DAY938 with the PmeI-digested plasmids pDDB343 and pDDB353, respectively.

Strains DAY1120, DAY1121, DAY1124, DAY1125, and DAY1321, which contain the *TOR1-1* allele, were generated as previously described, with minor modifications (13). Briefly, primers TOR1 A-C and TOR1 A-C Rev (Table 2) were annealed and used for transformation. Transformants were selected on yeast extract-peptone-dextrose (YPD) supplemented with 100 nM rapamycin. Rapamycin-resistant clones were screened by the NheI digestion of a PCR product amplified with primers JOHE6247 and JOHE6248 and were verified by sequencing (13). DAY1255 then was generated by transforming DAY1120 with PmeI-digested pDDB343. The correct genotype of all of the mutants carrying *TOR1-1* and/or *tor1*::*HIS1* alleles was verified by the Southern blotting of the NheI/NcoI or NheI/PvuII digestion of genomic DNA (data not shown) and using PCR products amplified with primers TOR1 probe 5′ and TOR1 probe 3′ or TOR1 probe 5′-2 and TOR1 probe 3′ as probes (Table 2). The probes were radiolabeled with [α-^{32}P]dCTP using the Prime-a-Gene labeling system (Promega).

The *mds3*Δ/Δ *sit4*Δ/Δ mutant DAY1233 was constructed by sequentially deleting both *MDS3* alleles from the *sit4*Δ/Δ strain DAY972, using the *mds3*::*URA3-dpl200* disruption cassette amplified with the primers MDS3 5DR and MDS3 3DR described above. The *URA3-dpl200* marker was recycled by growing the cells in synthetic complete (SC) medium supplemented with 5-fluoroorotic acid (5-FOA).

The *mds3*Δ/Δ *rbp1*Δ/Δ mutant DAY1239 was constructed by sequentially deleting both *MDS3* alleles from the *rbp1*Δ/Δ strain DAY1230. Strain DAY1230 was generated by recycling the *MX3*::*URA3R*::*MX3* marker in strain DAY1223 (13) in 5-FOA. The *mds3*::*URA3-dpl200* disruption cassette was amplified with primers MDS3 5DR and MDS3 3DR described above, and the *URA3-dpl200* marker was recycled by growing the cells in 5-FOA.

DAY1234, which contains a functional *SIT4*–c-*Myc* allele, was constructed by introducing a C-terminal MYC-T$_{ADH1}$-*URA3* cassette. The MYC-T$_{ADH1}$-*URA3* cassette was PCR amplified with primers Sit4 MYC Nter 5′ and Sit4 MYC URA3 Nter 3′ from plasmid pDDB372 (pMG1095 [30]) (Table 2). The correct integration of the cassette was verified by PCR using primers 5′ Myc detect and Sit4 3′ detect (Table 2) and by Western blotting using anti-Myc antibody (R950-25; Invitrogen).

To construct the strains for the coimmunoprecipitation studies, DAY1234 was transformed with NheI-digested pDDB499 (*MDS3-HA*) to generate strain DAY1236. DAY1234 also was transformed with PmeI-digested pDDB353 (untagged *MDS3* vector) to generate the control strain DAY1235.

The *MDS3* complementation vector pDDB353 was constructed as follows. Plasmid pDDB343 was generated by replacing the NruI restriction site in pDDB78 with a PmeI site. Primers DDB78 PmeIxNruI 5′ and DDB78 PmeIxNruI 3′ were annealed and used for the gap repair of the NruI-digested pDDB78 through *in vivo* recombination in *trp* mutant *S. cerevisiae* strain L40 to generate plasmid pDDB343. Wild-type *MDS3* sequence with flanking promoter and terminator sequences was amplified in two high-fidelity PCRs (*Pfu* turbo DNA polymerase; Stratagene) using primer pairs MDS3 5comp new and MDS3 5-2comp new as well as MDS3 3comp new-2 and MDS3 3-2comp new-2 from strain BWP17 genomic DNA (Table 2). Both PCR products were transformed with EcoRI/NotI-double digested pDDB343 into strain L40 to produce plasmid pDDB353 through *in vivo* recombination. The complete open reading frame (ORF) of *MDS3* was verified by sequencing.

The *MDS3-HA*-tagged vector DDB499 was constructed as follows. To find a location in *MDS3*'s ORF that could be tagged without affecting Mds3 function, we performed the random mutagenesis of *MDS3* using the GPS-LS linker scanning system (New England BioLabs). An NheI/AhdI fragment from plasmid DDB353 containing *MDS3* and its flanking regions was cloned into an NheI/AhdI-digested pGEM-T Easy vector (Promega), pDDB407, to generate plasmid pDDB408. pDDB408 was used as the substrate for random Tn*7* insertion mutagenesis. Plasmids carrying Tn*7* integrations within *MDS3*'s ORF were screened by PCR using primer S and primer N (Promega), MDS3null 5-detect, and MDS3null 3-detect (Table 2). The Tn*7* transposon was excised, and plasmids were religated, leaving a 15-bp insertion that contains a PmeI site. Plasmids containing mutated versions of *MDS3*'s ORF were AhdI/NdeI digested, subcloned into NotI/EcoRI-digested pDDB409 by *in vivo* recombination, and tested for the complementation of an *mds3*Δ/Δ mutant. Plasmid pDDB500, containing a 5-amino-acid insertion at amino acid 1051, was selected for hemagglutinin (HA) tagging. The HA tag was PCR amplified from plasmid pDDB369 (pMG1921 [30]) using primers MDS3-HA 56 5′ and MDS3-HA 56 3′ (Table 2) and *in vivo* recombined into the PmeI-digested plasmid pDDB500 using *S. cerevisiae* L40 strain to generate pDDB499, which inserts HA after residue 1056. pDDB499 rescued the *mds3*Δ/Δ mutant, indicating that Mds3 tolerated the addition of the 29 amino acids of the 3× HA tag.

Plasmid pDDB409 was constructed by replacing the unique NruI site in pDDB78 with a 26-bp linker containing NgoMIV-KpnI-NheI restriction sites. The 26-bp primers NgoMIV KpnI NheI × NruI 5′ and NgoMIV KpnI NheI × NruI 3′ were annealed and ligated in NruI-digested pDDB78 to give pDDB409.

Media and growth conditions. *C. albicans* was grown routinely at 30°C in YPD (2% Bacto peptone, 2% dextrose, 1% yeast extract). For the selection of Ura$^+$, His$^+$, or Trp$^+$ transformants, synthetic medium without uridine, histidine, or tryptophan was used (0.17% yeast nitrogen base without ammonium sulfate [Q-BioGene], 0.5% ammonium sulfate, 2% dextrose, and supplemented with a

TABLE 1. *C. albicans* and *S. cerevisiae* strains used in this study

Strain	Parent/ background	Genotype	Reference or source
DAY1 (BWP17)	SC5314	*ura3*::λ*imm434*/*ura3*::λ*imm434 his1*::*hisG*/*his1*::*hisG arg4*::*hisG*/*arg4*::*hisG*	78
DAY25	BWP17	*ura3*::λ*imm434*/*ura3*::λ*imm434 HIS1*::*his1*::*hisG*/*his1*::*hisG arg4*::*hisG*/*arg4*::*hisG rim101*::*ARG4*/*rim101*::*URA3*	15
DAY185	DAY286	*ura3*::λ*imm434*/*ura3*::λ*imm434 HIS1*::*his1*::*hisG*/*his1*::*hisG ARG4*::*URA3*::*arg4*::*hisG*/*arg4*::*hisG*	15
DAY286	BWP17	*ura3*::λ*imm434*/*ura3*::λ*imm434 his1*::*hisG*/*his1*::*hisG ARG4*::*URA3*::*arg4*::*hisG*/*arg4*::*hisG*	78
DAY414 (L40)	*S. cerevisiae*	*MAT*α *his3*Δ*200 trp1-901 leu2-3,-112 ade2 LYS2*::$(lexAop)_4$-*HIS3 URA3*::$(lexAop)_8$-*lacZ GAL4*	74
DAY415 (VIC1)[a]	BWP17	*ura3*::λ*imm434*/*ura3*::λ*imm434 his1*::*hisG*/*his1*::*hisG arg4*::*hisG*/*arg4*::*hisG mds3*::*ARG4*/*MDS3*	18
DAY439 (VIC25)	BWP17	*ura3*::λ*imm434*/*ura3*::λ*imm434 HIS1*::*his1*::*hisG*/*his1*::*hisG arg4*::*hisG*/*arg4*::*hisG mds3*::*Tn7*::*URA3*/*mds3*::*Tn7*::*UAU1*	18
DAY938	BWP17	*ura3*::λ*imm434*/*ura3*::λ*imm434 his1*::*hisG*/*his1*::*hisG arg4*::*hisG*/*arg4*::*hisG mds3*::*ARG4*/*mds3*::*URA3-dpl200*	This study
DAY956	DAY938	*ura3*::λ*imm434*/*ura3*::λ*imm434 his1*::*hisG*/*his1*::*hisG arg4*::*hisG*/*arg4*::*hisG mds3*::*ARG4*/*mds3*::*dpl200*	This study
DAY971 (CM02)	RM1000	*ura3*::λ*imm434*/*ura3*::λ*imm434 his1*::*hisG*/*his1*::*hisG sit4*::*HIS1*/*sit4*::*URA3*	46
DAY972 (CM20)	RM1000	*ura3*::λ*imm434*/*ura3*::λ*imm434 his1*::*hisG*/*his1*::*hisG sit4*::*HIS1*/*sit4*::*dpl200*	46
DAY973 (CM22)	RM1000	*ura3*::λ*imm434*/*ura3*::λ*imm434 his1*::*hisG*/*his1*::*hisG sit4*::*HIS1*/*sit4*::*dpl200* [*pVEC1*::*SIT4*]	46
DAY1109 (CDH107)	CAI4	*ura3*::λ*imm434*/*ura3*::λ*imm434 ras1*::*hisG-URA3-hisG*/*ras1*::*hisG*	45
DAY1118	DAY938	*ura3*::λ*imm434*/*ura3*::λ*imm434 HIS1*::*his1*::*hisG*/*his1*::*hisG arg4*::*hisG*/*arg4*::*hisG mds3*::*ARG4*/*mds3*::*URA3-dpl200*	This study
DAY1119	DAY938	*ura3*::λ*imm434*/*ura3*::λ*imm434 HIS1*::*MDS3*::*his1*::*hisG*/*his1*::*hisG arg4*::*hisG*/*arg4*::*hisG mds3*::*ARG4*/*mds3*::*URA3-dpl200*	This study
DAY1120	DAY938	*ura3*::λ*imm434*/*ura3*::λ*imm434 his1*::*hisG*/*his1*::*hisG arg4*::*hisG*/*arg4*::*hisG mds3*::*ARG4*/*mds3*::*URA3-dpl200 TOR1-1*/*TOR1*	This study
DAY1121	DAY185	*ura3*::λ*imm434*/*ura3*::λ*imm434 HIS1*::*his1*::*hisG*/*his1*::*hisG ARG4*::*URA3*::*arg4*::*hisG*/*arg4*::*hisG TOR1-1*/*TOR1*	This study
DAY1122	DAY938	*ura3*::λ*imm434*/*ura3*::λ*imm434 his1*::*hisG*/*his1*::*hisG arg4*::*hisG*/*arg4*::*hisG mds3*::*ARG4*/*mds3*::*URA3-dpl200 TOR1*/*tor1*::*HIS1*	This study
DAY1123	DAY286	*ura3*::λ*imm434*/*ura3*::λ*imm434 his1*::*hisG*/*his1*::*hisG ARG4*::*URA3*::*arg4*::*hisG*/*arg4*::*hisG TOR1*/*tor1*::*HIS1*	This study
DAY1124	DAY1122	*ura3*::λ*imm434*/*ura3*::λ*imm434 his1*::*hisG*/*his1*::*hisG arg4*::*hisG*/*arg4*::*hisG mds3*::*ARG4*/*mds3*::*URA3-dpl200 TOR1-1*/*tor1*::*HIS1*	This study
DAY1125	DAY1123	*ura3*::λ*imm434*/*ura3*::λ*imm434 his1*::*hisG*/*his1*::*hisG ARG4*::*URA3*::*arg4*::*hisG*/*arg4*::*hisG TOR1-1*/*tor1*::*HIS1*	This study
DAY1208 (MWY64)	SK1	*MAT***a**/*MAT*α *his3*/*his3 leu2*::*hisG*/*leu2*::*hisG trp1*::*hisG*/*trp1*::*hisG lys2*/*lys2 ura3*/*ura3 ho*::*LYS2*/*ho*::*LYS2*	54
DAY1209 (CMY164)	SK1	*MAT***a**/*MAT*α *mds3*::*URA3*/*mds3*::*URA3 his3*/*his3 leu2*::*hisG*/*leu2*::*hisG trp1*::*hisG*/*trp1*::*hisG lys2*/*lys2 ura3*/*ura3 ho*::*LYS2*/*ho*::*LYS2*	54
DAY1210 (CMY165)	SK1	*MAT***a**/*MAT*α *pmd1*::*LEU2*/*pmd1*::*LEU2 his3*/*his3 leu2*::*hisG*/*leu2*::*hisG trp1*::*hisG*/*trp1*::*hisG lys2*/*lys2 ura3*/*ura3 ho*::*LYS2*/*ho*::*LYS2*	54
DAY1211 (CMY166)	SK1	*MAT***a**/*MAT*α *mds3*::*URA3*/*mds3*::*URA3 pmd1*::*LEU2*/*pmd1*::*LEU2 his3*/*his3 leu2*::*hisG*/*leu2*::*hisG trp1*::*hisG*/*trp1*::*hisG lys2*/*lys2 ura3*/*ura3 ho*::*LYS2*/*ho*::*LYS2*	54
DAY1223 (YAG171)	CAI4	*ura3*::λ*imm434*/*ura3*::λ*imm434 rbp1*::*MX3*/*rbp1*::*CaURA3MX3R*	13
DAY1230	DAY1223	*ura3*::λ*imm434*/*ura3*::λ*imm434 rbp1*::*MX3*/*rbp1*::*MX3*	This study
DAY1231	DAY972	*ura3*::λ*imm434*/*ura3*::λ*imm434 his1*::*hisG*/*his1*::*hisG sit4*::*HIS1*/*sit4*::*dpl200 MDS3*/*mds3*::*URA3-dpl200*	This study
DAY1232	DAY1231	*ura3*::λ*imm434*/*ura3*::λ*imm434 his1*::*hisG*/*his1*::*hisG sit4*::*HIS1*/*sit4*::*dpl200 MDS3*/*mds3*::*dpl200*	This study
DAY1233	DAY1232	*ura3*::λ*imm434*/*ura3*::λ*imm434 his1*::*hisG*/*his1*::*hisG sit4*::*HIS1*/*sit4*::*dpl200 mds3*::*URA3-dpl200*/*mds3*::*dpl200*	This study
DAY1234	DAY1	*ura3*::λ*imm434*/*ura3*::λ*imm434 his1*::*hisG*/*his1*::*hisG arg4*::*hisG*/*arg4*::*hisG SIT4*/*SIT4-Myc-URA3*	This study
DAY1235	DAY1234	*ura3*::λ*imm434*/*ura3*::λ*imm434 HIS1*::*MDS3*::*his1*::*hisG*/*his1*::*hisG arg4*::*hisG*/*arg4*::*hisG SIT4*/*SIT4-Myc-URA3*	This study
DAY1236	DAY1234	*ura3*::λ*imm434*/*ura3*::λ*imm434 HIS1*::*MDS3-HA*::*his1*::*hisG*/*his1*::*hisG arg4*::*hisG*/*arg4*::*hisG SIT4*/*SIT4-Myc-URA3*	This study
DAY1237	DAY1230	*ura3*::λ*imm434*/*ura3*::λ*imm434 rbp1*::*MX3*/*rbp1*::*MX3 MDS3*/*mds3*::*URA3dpl200*	This study
DAY1238	DAY1237	*ura3*::λ*imm434*/*ura3*::λ*imm434 rbp1*::*MX3*/*rbp1*::*MX3 MDS3*/*mds3*::*dpl200*	This study
DAY1239	DAY1238	*ura3*::λ*imm434*/*ura3*::λ*imm434 rbp1*::*MX3*/*rbp1*::*MX3 mds3*::*dpl200*/*mds3*::*URA3dpl200*	This study
DAY1255	DAY1120	*ura3*::λ*imm434*/*ura3*::λ*imm434 HIS1*::*his1*::*hisG*/*his1*::*hisG arg4*::*hisG*/*arg4*::*hisG mds3*::*ARG4*/*mds3*::*URA3-dpl200 TOR1-1*/*TOR1*	This study
DAY1321	DAY972	*ura3*::λ*imm434*/*ura3*::λ*imm434 his1*::*hisG*/*his1*::*hisG sit4*::*HIS1*/*sit4*::*dpl200 TOR1-1*/*TOR1*	This study

[a] Parent of VIC3 (*mds3*::*ARG4*/*mds3*::*URA3*). VIC1 was not described by Davis et al. (6).

TABLE 2. Primers used in this study

Name	Sequence[a] (5′ to 3′)	Reference
MDS3 5DR	TCTTAAGGCACAAGTTATTGGCTTGACGTAGAAGTTTGCAAAGATTTTCACAATATC ATGTTTCCCAGTCACGACGTT	18
MDS3 3DR	ACCAATATAACGTGAATATACACCCCTATATTATTATCTTTTAATCCTGTTAACAATC CGTGGAATTGTGAGCGGATA	18
MDS3null 5-detect	GTGTCCCAATTTTGTCTAGC	This study
MDS3null 3-detect	TGCGGAAGAACTGTAAACCC	This study
TOR1 5DR	AGTAGATAATACTTCTAACTTTGATAGTAACATTAACGAAGAAAAACAAATCATTA ATCATTTCCCAGTCACGACGTT	This study
TOR1 3DR	TATTTCCCTTTATAAAATAGTTACACATACCATACTTAACGACACATGACGATACTC AACGTGGAATTGTGAGCGGATA	This study
TOR1 5′detect-2	TCTCTTAGTTGTTGAGTGGC	This study
TOR1 3′detect	AAATTTCCTTCCAAACCTGC	This study
TOR1 A-C	GTTTTATGGCACGAACAATGGCACGATGCTTTGGAAGATGCT**C**GCAGGTTTTTCTTT GGTGAACACAACACAGAAAAGATG	This study
TOR1 A-C Rev	CATCTTTTCTGTGTTGTGTTCACCAAAGAAAAACCTGC**G**AGCATCTTCCAAAGCATC GTGCCATTGTTCGTGCCATAAAAC	This study
JOHE6247	GGCAAGGTGTTTCTTGAAGC	13
JOHE6248	TACTTCTTGATTCGCGATAGC	13
DDB78 PmeIxNruI 5′	ATAATCAAAATGAGTCAATTCTCACAACCGCTCGGTTTAAACCGACACGTTTCAACG AAATGGCCTCCCCTACCA	This study
DDB78 PmeIxNruI 3′	TGGTAGGGGAGGCCATTTCGTTGAAACGTGTCGGTTTAAACCGAGCGGTTGTGAGA ATTGACTCATTTTGATTAT	This study
MDS3 5comp new	AAGCTCGGAATTAACCCTCACTAAAGGGAACAAAAGCTGGTGGTCTTTAATTGGGC GGAC	This study
MDS3 3comp new-2	ACGACGGCCAGTGAATTGTAATACGACTCACTATAGGGCGGTTCTCGATTAGTCCA TTGACCTGCC	This study
MDS3 5-2comp new	GATGGAGAGATGGGGACAGC	This study
MDS3 3-2comp new-2	TTTGTCATGTTCACCCGACG	This study
TOR1 probe 5′	ATGAAATAGTGAGTCGCTCC	This study
TOR1 probe 5′-2	CTTTTGTTGCAAATGGCACC	This study
TOR1 probe 3′	CTACCAAGCATAATGGGGTC	This study
RPS26A 5′	CAAGAAAGGTAGAGGTCACG	This study
RPS26A 3′	TTTAGCAGCATCTTGTGGAG	This study
CDC19 5′ a	CTTCAATGTTGAAACTGTTCC	This study
CDC19 3′ b	AACAGTGTTAGAGTGACCAG	This study
GAP2 5′	TTATTGTACAGTTATGTCCC	This study
GAP2 3′	CCAGCGAAAGCAAAGGCAGC	This study
TEF1-5′	ATAGTCATAATCAATCATGGGT	16
TEF1-3′	CTTACATAATATTCAACTAGC	16
Sit4 MYC N-ter 5′	GATGGTGACTTATCAGTCAAGAACAATGCCAACAAACAACAAAGAAGTGATTATTT TTTGCGGATCCCCGGGTTAATTAA	This study
Sit4 MYC URA3 N-ter 3′	GCATCATTAAGGGATTTGAAAAAAAAAGATATAAATATAAAAATCATTCATCATTC GTTATCTAGAAGGACCACCTTTGATTG	This study
5′ Myc detect	GAACAAAAATTAATTTCTGAAGAAGATTTA	This study
Sit4 3′ detect	TGGTTTGTATGTGTAAGTCC	This study
NgoMIV KpnI NheI x NruI 5′	TAGCCGGCTGGGTACCCGGCTAGCCA	This study
NgoMIV KpnI NheI x NruI 3′	TGGCTAGCCGGGTACCCAGCCGGCTA	This study
MDS3-HA-56 5′	AGCTTCGAAAATTCATGCAGATAGTGTCGCAATGTCTTTGTACCCATACGATGTTCC TGAC	This study
MDS3-HA-56 3′	AAGCATTTCGCAGATTATTACTTGTAACCGATTTCTTTTTGTAATCTGGAACGTCAT ATGG	This study

[a] The sequences underlined indicate the restriction site created. Sequences in boldface and underlined indicate point mutations.

dropout mix containing amino and nucleic acids except those necessary for the selection [1]). Media were buffered at the indicated pH using 150 mM HEPES. The assays for filamentation in the presence of rapamycin were performed in M199 medium (Gibco BRL) buffered at pH 8 and SLAD (0.17% yeast nitrogen base without ammonium sulfate [Q-BioGene], 50 μM ammonium sulfate, 2% dextrose). Filamentation assays were conducted at 37°C except as indicated. Rapamycin (LC Laboratories) was added to the media at the indicated concentrations from a stock solution in 90% ethanol–10% Tween 20. For liquid assays of filamentation in the presence of rapamycin, strains were pregrown in liquid YPD at 30°C, pelleted, resuspended in an equal volume of phosphate-buffered saline (PBS), and diluted 1:100 in M199, pH 8, supplemented with rapamycin or solvent alone. Samples were incubated for 6 h. To determine the percentage of hypha-producing cells, samples were gently sonicated and quantified under the microscope. The values reported represent the averages from at least two independent experiments, in which every strain was tested at least in duplicate. For filamentation in serum, strains were pregrown in liquid YPD at 30°C, diluted 1:100 in liquid YPD supplemented with 10% fetal bovine serum (Gibco), and incubated at 37°C for 5.5 h. For growth assays in the presence of rapamycin, strains were pregrown in liquid YPD at 30°C, diluted in PBS to an optical density at 600 nm (OD_{600}) of 1.6, and then serially diluted 5-fold in PBS, spotted on YPD or Spider medium (1% mannitol, 1% nutrient broth, 0.2% K_2HPO_4, pH 7.2, before being autoclaved [47]), supplemented with rapamycin or solvent alone, and incubated at 30°C for 2 or 3 days. All media except that for the selection of Ura^+ transformants were supplemented with 80 μg/ml uridine. For solid media, 2% Bacto agar was added, except for Spider medium, which used 1.35% Bacto agar.

Microarray analysis. Strains were pregrown in YPD at 30°C, pelleted, washed with M199 (pH 4 or pH 8) medium, diluted 40-fold in prewarmed M199 (pH 4

TABLE 3. Results of the transcriptional profiling of the wild-type, *mds3*Δ/Δ, and *rim101*Δ/Δ *C. albicans* strains

Condition	No. of ORFs with ≥2-fold difference		No. of ORFs expressed more in the mutant		No. of ORFs expressed less in the mutant	
	*mds3*Δ/Δ	*rim101*Δ/Δ[a]	*Mds3*Δ/Δ	*rim101*Δ/Δ[a]	*mds3*Δ/Δ	*rim101*Δ/Δ[a]
WT pH 8 versus mutant pH 8	337	186	146	55	191	131
WT pH 4 versus mutant pH 4	76	7	37	1	39	6
ORFs ≥2-fold difference at pH 8 and 4	50	6				
Total	363	187				

[a] Data are from reference 6.

or pH 8), and incubated for 4 h at 37°C with agitation. Procedures for RNA extraction, microarray construction, and analysis have been described previously (6). Corrected *P* values for the gene ontology (GO) categories were obtained using the GO term-finder algorithms available through the Candida Genome Database website (www.candidagenome.org) in February 2009.

Northern blot analysis. Cells were grown overnight in YPD at 30°C. The following day, cells were washed with M199 medium at either pH 4 or 8 and diluted 40-fold into fresh M199 (pH 4 or 8) medium containing solvent or 5 nM rapamycin and incubated for 4 h at 37°C with agitation. For Spider medium experiments, cells were diluted 1:40 in Spider medium containing solvent or 5 nM rapamycin and incubated for 4 h at 37°C with agitation. For YPD medium experiments, cells were grown as described previously (4). Briefly, YPD overnight cultures were inoculated into YPD at an OD of ~0.1. Cells were grown 5 h at 30°C, followed by 1 h at 30°C with solvent or 20 nM rapamycin. Cells then were harvested and frozen in a dry ice-ethanol bath. RNA extraction and Northern blot procedures were previously described (6), except that the PCR products for the probes were purified with a PCR purification kit (Qiagen). RNA concentration was measured using a NanoDrop spectrophotometer ND-1000. Probes for *ECE1* and *HWP1* were described previously (18).

Statistical analysis. Statistical analysis for data shown in Table 5 was performed using the software SAS 9.13 (SAS Institute Inc.). We used repeated analyses of covariance (ANCOVA) (assuming different variances for each strain) and included the day of the experiment as the covariate. We tested for strain-treatment interaction and compared specific strain treatment effects after adjusting for multiple comparisons using Bonferroni's correction (14 comparisons were made; significant *P* values were <0.05/14, or 0.0036).

Microscopy. Pictures of colonies were taken using a Canon Powershot A560 digital camera on a Zeiss Opton microscope. Images of liquid cultures were captured using a Zeiss Axio camera, Axiovision 4.6.3 software (Zeiss), and a Zeiss AxioImager fluorescence microscope. All images were processed with Adobe Photoshop 7.0 software.

Protein purification and Western blot analysis. Overnight cultures of *C. albicans* were diluted 200-fold into M199 (pH 8) or YPD medium and grown for 4 to 5 h at 30°C. Cells were pelleted and resuspended in ice-cold radioimmunoprecipitation assay (RIPA) buffer (50 mM Tris, pH 8, 150 mM NaCl, 1% NP-40, 3 mM EDTA, 0.5% deoxycholate, 0.1% sodium dodecyl sulfate [SDS]) containing 1 μg/ml leupeptin, 2 μg/ml aprotinin, 1 μg/ml pepstatin, 0.1 mM phenylmethylsulfonyl fluoride, and 10 mM dithiothreitol and lysed by vortexing with acid-washed glass beads for 1 h at 4°C. Cell lysates were pelleted and supernatants stored at −80°C. For Western blot assays of the input, ~1.25 mg of total protein was resuspended in 2× SDS gel-loading buffer (100 mM Tris-Cl, pH 6.8; 200 mM dithiothreitol; 4% SDS; 0.1% bromophenol blue; 20% glycerol), boiled at 95 to 100°C for 3 min, and run in an 8% SDS-PAGE gel. Proteins were transferred to nitrocellulose and blocked in 6% nonfat milk in TBS-T (50 mM Tris, pH 7.6, 150 mM NaCl, 0.1% Tween 20). Blots were incubated with anti-HA (F-7 probe; Santa Cruz) or anti-Myc (Invitrogen) at 1:1,000 or 1:5,000 dilution, respectively, in 6% nonfat milk TBS-T, washed in TBS-T, and incubated with anti-mouse antibody–horseradish peroxidase (GE Healthcare) at 1:5,000 in 6% nonfat milk in TBS-T. Blots were washed in TBS-T, incubated with ECL reagent (GE Healthcare), and exposed to film.

Coimmunoprecipitations. Mds3-HA was immunoprecipitated as follows. Total protein (~18 mg) was incubated overnight at 4°C with 20 μl of slurry anti-HA agarose beads (Sigma-Aldrich). Beads were washed four times with Tris-buffered saline (25 mM Tris, 0.15 M NaCl, pH 7.2, 0.05% Tween). Protein was eluted from the beads by resuspension in 50 μl 2× SDS gel loading buffer and boiling for 5 min. The products of two immunoprecipitations were combined, separated by SDS-PAGE, and analyzed by Western blotting.

RESULTS

Mds3 affects TOR-dependent gene expression. Mds3 acts in parallel to the Rim101 pathway to promote the yeast-to-hypha morphogenetic switch in response to neutral-alkaline pH (18). To gain insights into how Mds3 contributes to pH responses, we determined the transcriptional profiles of wild-type, *rim101*Δ/Δ, and *mds3*Δ/Δ strains grown at pH 8 and 4 (see Table S1 in the supplemental material). The microarray experiments for all three strains were performed concomitantly, but the results for the wild-type and *rim101*Δ/Δ strains, including the microarray methodology and validation, have been reported previously (6).

Since Mds3 and Rim101 are required for growth and morphogenesis at alkaline pH, we expected that most transcriptional differences observed between the mutant strains and the wild type would occur at pH 8. Indeed, ~80% (287) and ~95% (180) of all differentially expressed ORFs in the *mds3*Δ/Δ and *rim101*Δ/Δ mutants, respectively, occurred at pH 8 (Table 3) (6). Further, because the *mds3*Δ/Δ and *rim101*Δ/Δ mutants are defective for filamentation at alkaline pH, we expected to find common changes in the expression of hypha-associated genes. In fact, the expression of genes associated with cell wall/filamentation were decreased in the *mds3*Δ/Δ and *rim101*Δ/Δ mutants relative to that of the wild-type strain (Table 4) (6). Thus, *mds3*Δ/Δ and *rim101*Δ/Δ mutants share similar transcriptional defects, as predicted based on their similar phenotypes at alkaline pH.

However, as Mds3 and Rim101 act in parallel, we also expected to find differences between the transcriptional profiles of these mutants relative to that of the wild-type strain. Indeed, several fundamental differences were observed. First, the *mds3*Δ/Δ mutant affected the expression of twice as many genes as did the *rim101*Δ/Δ mutant (363 versus 187 total ORFs, respectively) (Table 3). Approximately 60% (229) of the genes affected by the *mds3*Δ/Δ mutant were not pH regulated in wild-type cells, compared to ~38% (72) of the genes in the *rim101*Δ/Δ mutant (Fig. 1). Second, the *mds3*Δ/Δ mutant affected the expression of more genes at pH 4 than the *rim101*Δ/Δ mutant (76 versus 7 genes, respectively) (Table 3). Third, in the *rim101*Δ/Δ mutant, ~70% of the differentially expressed genes showed reduced expression, suggesting that Rim101 primarily functions as a positive regulator (Table 3). However, in the *mds3*Δ/Δ mutant, the differentially expressed genes showed increased and reduced expression at an ~50:50 ratio, demonstrating that Mds3 functions as both a positive and negative regulator (Table 3). Overall, these results suggest that

TABLE 4. GO categories of differentially expressed ORFs in the *mds3*Δ/Δ and *rim101*Δ/Δ mutants compared to those of wild-type *C. albicans*

GO category	No. of ORFs							
	Expressed more at pH 8		Expressed less at pH 8		Expressed more at pH 4		Expressed less at pH 4	
	*mds3*Δ/Δ	*rim101*Δ/Δ	*mds3*Δ/Δ	*rim101*Δ/Δ	*mds3*Δ/Δ	*rim101*Δ/Δ	*mds3*Δ/Δ	*rim101*Δ/Δ
Amino acid biosynthesis	11	1	8	4	6	0	3	3
Carbohydrate metabolism	15	3	8	3	8	0	0	0
Glycolysis	6	0	0	0	5	0	0	0
Translation	50	0	1	1	0	0	0	0
Initiation/elongation	6	0	0	0	0	0	0	0
rRNA/tRNA processing	5	0	0	0	0	0	0	0
Ribosome	37	0	1	0	0	0	0	0
Mitochondrial	2	0	0	1	0	0	0	0
Protein synthesis/folding	15	2	1	2	2	0	0	0
Protein degradation/autophagy	2	0	11	0	0	0	1	1
Vacuolar protein catabolic activity	0	0	5	0	0	0	0	1
Amino acid degradation/proteosome	2	0	3	0	0	0	1	0
Others	0	0	3	0	0	0	0	0
Lipid metabolism	4	1	10	4	1	0	0	0
Permeases/transport	9	9	33	25	2	0	13	0
Amino acid transport	0	2	12	5	0	0	4	0
Hexose transport	4	0	4	1	1	0	1	0
Other transport	5	7	17	19	1	0	8	0
DNA replication/transcription	7	1	2	0	1	0	2	0
Transcription factor/signal transduction	2	2	13	4	3	0	5	0
Electron transport	2	0	3	0	1	0	0	0
Cell wall/filamentation	4	2	22	17	1	1	3	2
Other	10	7	19	23	4	0	4	0
Unknown/novel	15	27	60	48	8	0	8	0
Total ORFs	146	55	191	131	37	1	39	6

Mds3 has broader effects on gene expression than Rim101 and that Mds3 has roles in addition to adaptation to environmental pH.

To identify potential biological functions for Mds3, we analyzed the Mds3-dependent genes by gene ontology (GO). Of the genes upregulated in the *mds3*Δ/Δ mutant compared to the wild-type strain, the GO categories of glycolysis and translation were significantly overrepresented ($P < 1.1E{-}04$ and $P < 5.3E{-}15$, respectively) (Table 4; also see Table S1 in the supplemental material). In wild-type cells, most of the ORFs in translation were expressed preferentially at pH 8 compared to pH 4 (6). This suggests that the loss of Mds3 enhances these gene expression changes observed in wild-type cells at alkaline pH. Of the genes downregulated in the *mds3*Δ/Δ mutant compared to the wild-type strain, the GO categories of amino acid transport and vacuolar protein catabolic activity ($P < 4.7E{-}04$ and $P < 1.6E{-}03$, respectively) were significantly overrepresented (Table 4). Further, the categories of amine transport, lytic vacuole, glycolysis, and translation also were overrepresented when this analysis was done in the *mds3*Δ/Δ mutant exclusively for genes that are pH independent (data not shown). These results show that Mds3 affects a variety of distinct processes, including growth and starvation responses, and supports the idea that Mds3 has functions in pH-independent processes.

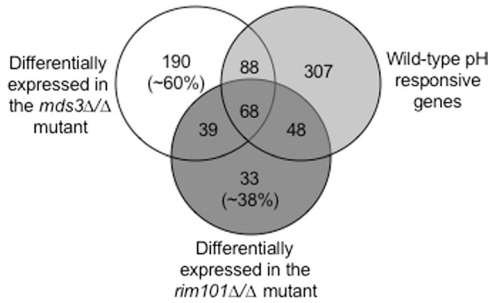

FIG. 1. VENN diagram of wild-type and Mds3- and Rim101-dependent genes. Differentially expressed genes in the *mds3*Δ/Δ or *rim101*Δ/Δ mutant versus expression in the wild-type strain were separated into pH-dependent and pH-independent groups depending on whether these ORFs were differentially expressed in wild-type cells in response to pH. Genes that were differentially regulated in the *mds3*Δ/Δ or *rim101*Δ/Δ mutant versus that in the wild-type strain at both pH 8 and 4 were included only once in this analysis. Numbers in parentheses represent the percentages of ORFs that are pH independent.

To corroborate the microarray results, we analyzed the expression of several *MDS3*-dependent ORFs by Northern blotting (Fig. 2A). In M199 pH 8 medium, the glycolysis gene *CDC19* and the translation genes *RPS26A* and *TEF1* were expressed ~2- to 4-fold more in the *mds3*Δ/Δ mutant than the wild-type or the complemented *mds3*Δ/Δ +*MDS3* strains (Fig. 2A, lanes 6 to 8). The increased expression of *CDC19*, *RPS26A*,

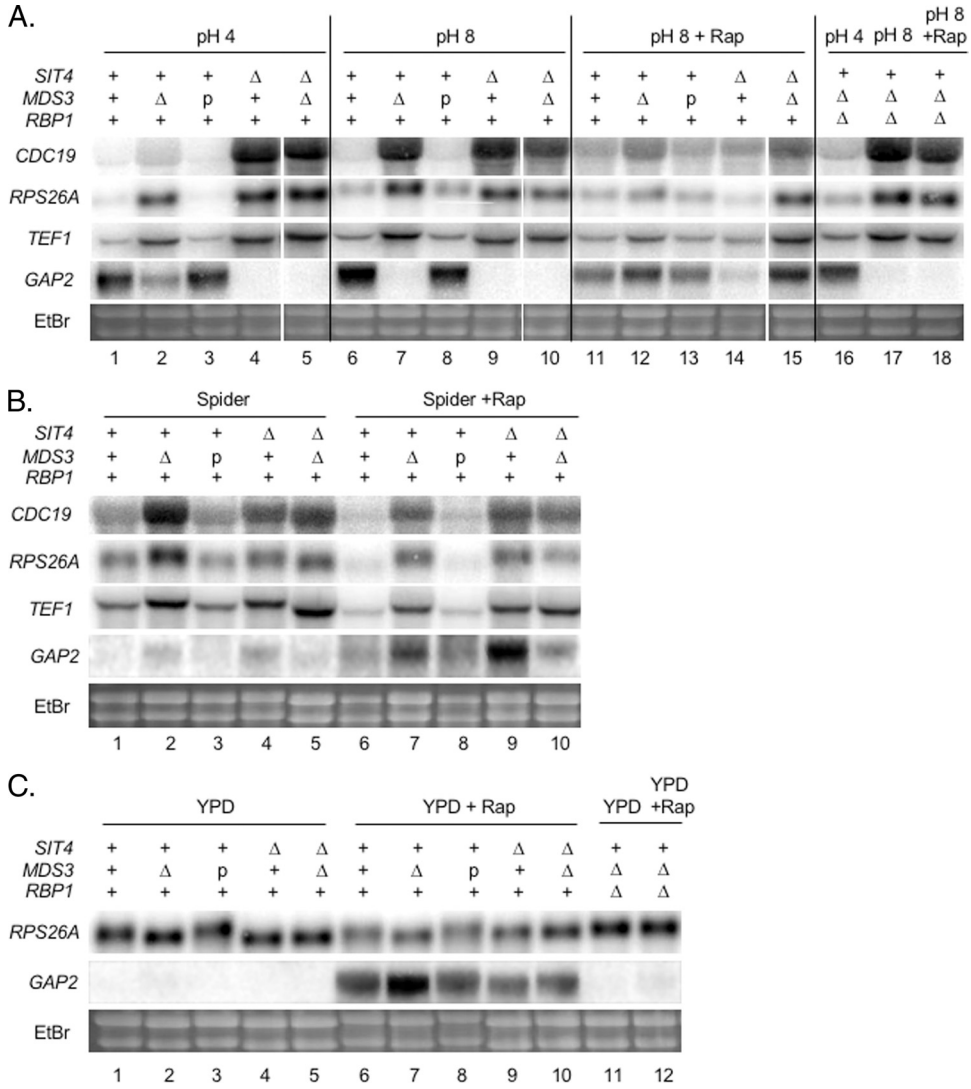

FIG. 2. Microarray validation by Northern blot analysis. Overnight YPD cultures of the wild-type (DAY185), *mds3*Δ/Δ (DAY1118), *mds3*Δ/Δ +*MDS3* (DAY1119), *sit4*Δ/Δ (DAY972), *sit4*Δ/Δ *mds3*Δ/Δ (DAY1233), and *mds3*Δ/Δ *rbp1*Δ/Δ (DAY1239) strains were diluted in prewarmed M199 pH 8 (A), Spider medium (B), or YPD (C) with or without rapamycin. Ethidium bromide staining was used as a loading control, because commonly used loading controls (*TEF1*, *ACT1*, or *TDH3*) varied in expression due to the mutations, the growth conditions, and/or the addition of rapamycin. *MDS3*, *SIT4*, and *RBP1* genotypes are noted above the samples: wild-type (+), deletion (Δ), or complemented (p).

and *TEF1* in the *mds3*Δ/Δ mutant also was observed at pH 4 (Fig. 2A, lanes 1 to 3), suggesting that Mds3 governs the expression of these genes in a pH-independent manner. *GAP2*, an amino acid transport gene, was reduced >2-fold in the *mds3*Δ/Δ mutant compared to the wild-type or the *mds3*Δ/Δ +*MDS3*-complemented strain at both pH 4 and 8 (Fig. 2A, compare lanes 2 and 7 to lanes 1, 3, 6, and 8). These results establish the veracity of the microarray data and support the idea that Mds3 has pH-independent functions.

The GO categories affected by the *mds3*Δ/Δ mutant reflect processes that are transcriptionally regulated by the TOR pathway. During conditions of active growth, TOR positively regulates the expression of genes involved in ribosomal biogenesis, translation initiation and elongation, rRNA and tRNA synthesis, and glycolysis (79), and it negatively regulates the expression of genes involved in protein degradation and nitrogen catabolite repression (NCR) (4, 8, 35, 68). During starvation conditions, TOR is inactive and genes involved in translation and glycolysis are repressed, while genes involved in amino acid transport and vacuolar degradation are induced (4, 8, 35, 68). Since the *mds3*Δ/Δ mutant shows gene expression differences that reflect TOR activation, we hypothesized that Mds3 functions as a negative regulator of TOR.

To address the idea that Mds3 is a negative regulator of TOR, we predicted that rapamycin, a TOR inhibitor (36), would rescue the transcriptional defects associated with the *mds3*Δ/Δ mutant. Indeed, 5 nM rapamycin reduced the expression of *CDC19*, *RPS26A*, and *TEF1* and restored the expression of *GAP2* in *mds3*Δ/Δ mutant cells compared to that with solvent alone (Fig. 2A, compare lanes 6 to 8 and 11 to 13). Rapamycin did not appear to affect the expression of *RPS26A*, *TEF1*, and *GAP2* in the wild-type or *mds3*Δ/Δ +*MDS3* complemented

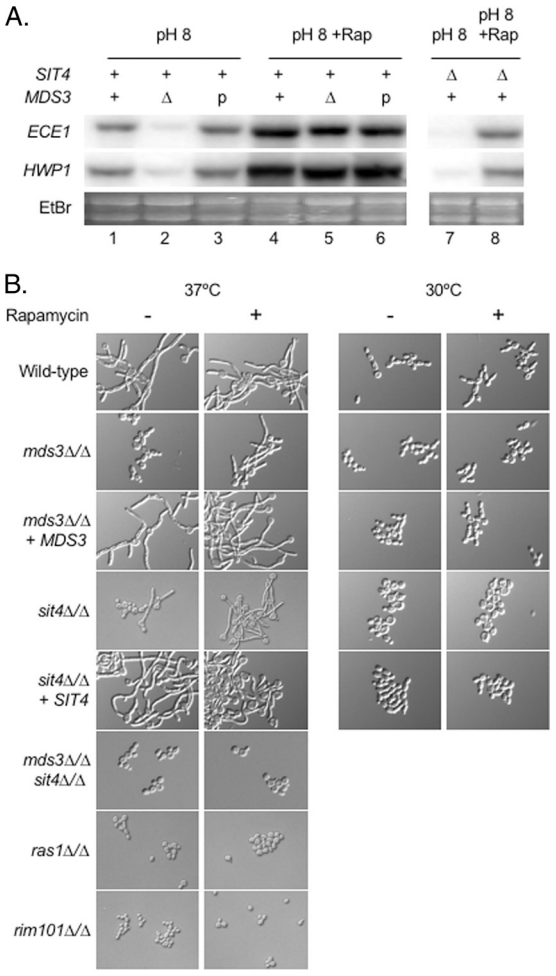

FIG. 3. Rapamycin rescues *mds3*Δ/Δ hypha-associated phenotypes. (A) Northern blots of hypha-associated genes performed on the wild-type (DAY185), *mds3*Δ/Δ (DAY1118), *mds3*Δ/Δ+*MDS3* (DAY1119), and *sit4*Δ/Δ (DAY972) strains grown for 4 h in M199, pH 8, supplemented with solvent or 5 nM rapamycin. *MDS3* and *SIT4* genotypes are noted above the samples: wild-type (+), deletion (Δ), or complemented (p). EtBr, ethidium bromide. (B) Overnight YPD cultures of the wild-type (DAY185), *mds3*Δ/Δ (DAY1118), *mds3*Δ/Δ +*MDS3* (DAY1119), *sit4*Δ/Δ (DAY972), *sit4*Δ/Δ +*SIT4* (DAY973), *mds3*Δ/Δ *sit4*Δ/Δ (DAY1233), *rim101*Δ/Δ (DAY25), and *ras1*Δ/Δ (DAY1109) strains were washed in PBS and diluted 1:100 in M199 liquid medium (pH 8) with or without 5 nM rapamycin. Cells were visualized under the microscope (40× magnification) after 6 h of incubation at 30 or 37°C.

strains; however, the expression of *CDC19* was increased ~2-fold compared to that in solvent alone. Regardless, in the presence of rapamycin the expression of *CDC19*, *RPS26A*, *TEF1*, and *GAP2* was quantitatively similar in wild-type, *mds3*Δ/Δ, and *mds3*Δ/Δ +*MDS3* cells. These results demonstrate that rapamycin restores the transcriptional defects due to the loss of Mds3 and corroborates the idea that Mds3 acts as a negative regulator of TOR.

In nutrient-poor Spider medium, we observed similar transcriptional results (Fig. 2B). The expression of *CDC19*, *RPS26A*, and *TEF1* was increased in the *mds3*Δ/Δ mutant compared to that of the wild type (Fig. 2B, compare lanes 1 and 2). Further, the addition of rapamycin reduced *CDC19*, *RPS26A*, and *TEF1* expression in the *mds3*Δ/Δ mutant-, wild-type-, and *mds3*Δ/Δ +*MDS3*-complemented strains. We noted that in Spider medium this rapamycin-dependent restoration of gene expression in the *mds3*Δ/Δ mutant was less than that observed in M199 pH 8 medium. We also found that in Spider medium, *GAP2* was expressed poorly in wild-type cells and expressed at greater levels in the absence of *MDS3*. The addition of rapamycin increased *GAP2* expression in all strains tested (Fig. 2B, compare lanes 1 to 5 with 6 to 10). These results support the idea that Mds3 acts in the TOR pathway and suggests that, not surprisingly, growth medium differences cause distinct effects.

Rapamycin rescues the *mds3*Δ/Δ mutant filamentation defect. The TOR pathway regulates fungal morphogenesis (14), and rapamycin triggers the expression of hypha-associated genes in *C. albicans* (4). Since Mds3 also regulates morphogenesis and is required for the expression of hypha-associated genes (18), we asked if rapamycin also rescues the expression of these genes in the *mds3*Δ/Δ mutant (Fig. 3A). Wild-type, *mds3*Δ/Δ, and *mds3*Δ/Δ +*MDS3* cells were incubated in M199 pH 8 medium at 37°C, which promotes hypha formation, with and without 5 nM rapamycin and analyzed the expression of the hypha-associated genes *HWP1* and *ECE1* (Fig. 3A). While the *mds3*Δ/Δ mutant showed little *HWP1* and *ECE1* expression, the addition of rapamycin completely restored this expression (Fig. 3A, compare lanes 2 and 5). We noted that rapamycin also led to the increased expression of *HWP1* and *ECE1* in both wild-type and *mds3*Δ/Δ +*MDS3* strains compared to that in solvent alone. Thus, rapamycin promotes the expression of hypha-associated genes and rescues the *mds3*Δ/Δ mutant defects in *HWP1* and *ECE1* expression.

Since rapamycin rescued the transcriptional defects associated with the loss of Mds3, we asked if rapamycin also could rescue the hyphal formation defect of the *mds3*Δ/Δ mutant. In liquid M199 pH 8 medium at 37°C, >90% of wild-type cells germinated to form hyphae; <5% of *mds3*Δ/Δ mutant cells germinated to form hyphae (Table 5 and Fig. 3B). However, the addition of 5 nM rapamycin partially restored hyphal formation in the *mds3*Δ/Δ mutant to 45% ($P < 0.0001$ compared to *mds3*Δ/Δ in solvent) (Fig. 3B and Table 5). Rapamycin also promoted hyphal formation in wild-type cells compared to formation in the solvent control ($P < 0.001$) (Table 5), and we noted that the hyphae formed in the wild-type, *mds3*Δ/Δ, and *mds3*Δ/Δ +*MDS3* strains in the presence of rapamycin appeared less branched than in solvent alone. Similar results were observed in liquid SLAD medium, a nutrient-poor medium, indicating that the effect of rapamycin is not spe-

TABLE 5. Hyphal formation in M199 pH 8 in the presence and absence of 5 nM rapamycin

Strain	Genotype	% of hyphae ± SEM	
		Solvent	Rapamycin
DAY185	*MDS3/MDS3 TOR1/TOR1*	88.5 ± 2.0	98.3 ± 2.0
DAY1121	*MDS3/MDS3 TOR1-1/TOR1*	85.0 ± 2.2	89.6 ± 2.2
DAY1123	*MDS3/MDS3 TOR1/*Δ	94.3 ± 0.7	98.5 ± 0.7
DAY1125	*MDS3/MDS3 TOR1-1/*Δ	90.6 ± 1.6	93.3 ± 1.6
DAY1118	*mds3*Δ/Δ *TOR1/TOR1*	2.9 ± 3.2	44.5 ± 3.2
DAY1120	*mds3*Δ/Δ *TOR1-1/TOR1*	5.0 ± 3.2	23.4 ± 3.2
DAY1122	*mds3*Δ/Δ *TOR1/*Δ	5.1 ± 1.8	37.3 ± 1.8
DAY1124	*mds3*Δ/Δ *TOR1-1/*Δ	0.9 ± 0.3	0.8 ± 0.3

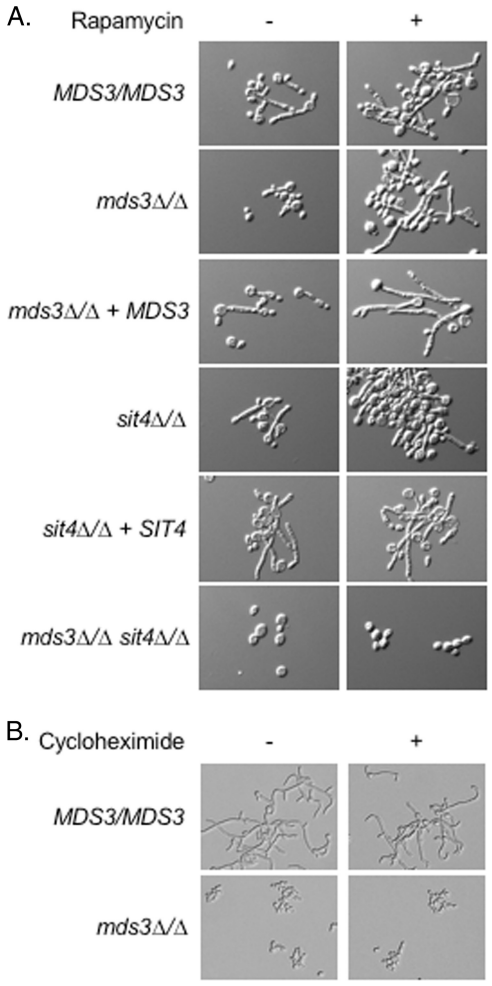

FIG. 4. Rapamycin rescues *mds3*Δ/Δ filamentation defects in nutrient-poor medium but cycloheximide does not. (A) Wild-type (DAY185), *mds3*Δ/Δ (DAY1118), *mds3*Δ/Δ +*MDS3* (DAY1119), *sit4*Δ/Δ (DAY971), *sit4*Δ/Δ +*SIT4* (DAY973), and *mds3*Δ/Δ *sit4*Δ/Δ (DAY1233) cells were grown for 6 h in SLAD liquid medium at 37°C with or without 10 nM rapamycin. (B) Wild-type (DAY185) and *mds3*Δ/Δ (DAY1118) cells were grown for 6 h in M199 liquid medium (pH 8) at 37°C with or without 100 μM cycloheximide. Similar results were observed with 150 and 250 μM cycloheximide, but higher concentrations inhibited the growth of all strains tested.

cific to M199 medium (Fig. 4A). These results demonstrate that rapamycin rescues the morphogenetic defect of the *mds3*Δ/Δ mutant.

We found that the effect of rapamycin on hyphal formation was not due to translational inhibition, because cycloheximide did not rescue the filamentation defect of the *mds3*Δ/Δ mutant (Fig. 4B). Further, rapamycin is not a constitutive inducer of hyphal formation, because rapamycin did not rescue the *mds3*Δ/Δ hyphal formation defect in M199 pH 8 medium at 30°C (Fig. 3B). These results demonstrate that rapamycin is not a constitutive inducer of hyphal formation and further support a link between Mds3 and TOR.

To determine if the effect of rapamycin was actually dependent on TOR, we introduced the rapamycin-resistant *TOR1-1* allele into the wild-type and *mds3*Δ/Δ strains and determined if rapamycin still promoted hyphal formation (7, 13). As expected, strains carrying a *TOR1-1* allele were resistant to rapamycin (Fig. 5A). In the absence of rapamycin, the *TOR1-1* allele did not affect hyphal formation in either the *MDS3*/*MDS3* or *mds3*Δ/Δ background (Fig. 5B and Table 5). However, in the presence of rapamycin, the *TOR1-1* allele prevented the rapamycin-induced hyphal formation observed in *MDS3*/*MDS3* cells (Table 5), and the hypha formed showed a branching pattern similar to that of the wild-type strain without rapamycin (Fig. 5B). Thus, in the *MDS3*/*MDS3* background rapamycin-mediated phenotypes are TOR dependent. In the presence of rapamycin, the *TOR1-1* allele reduced the rapamycin-induced hyphal formation observed in *mds3*Δ/Δ cells by ~50% (Table 5). We hypothesized that the semiresponsive effect of rapamycin in the *mds3*Δ/Δ *TOR1-1*/*TOR1* mutant was an attribute of the remaining wild-type *TOR1* allele. To address this possibility, we constructed *TOR1-1*/Δ strains in the *MDS3*/*MDS3* and *mds3*Δ/Δ backgrounds. The deletion of one copy of *TOR1* in either background had no significant effect on hyphal formation (Fig. 5B and Table 5), demonstrating that *TOR1* is not haploinsufficient. While the *MDS3*/*MDS3 TOR1-1*/Δ strain filamented similarly to the *MDS3*/*MDS3 TOR1-1*/*TOR1* strain in the presence or absence of rapamycin, the *mds3*Δ/Δ *TOR1-1*/Δ strain failed to filament in the presence or absence of rapamycin (Fig. 5B and Table 5). The *mds3*Δ/Δ *TOR1-1*/Δ mutant did form hyphae in serum, an *MDS3*-independent inducer of hyphal formation (data not shown and reference 18), demonstrating that the *mds3*Δ/Δ *TOR1-1*/Δ strain did not have an absolute block in hyphal formation. These results demonstrate that rapamycin rescues *mds3*Δ/Δ phenotypes through TOR.

We noted that in the absence of a wild-type copy of *TOR1*, the *TOR1-1* allele conferred growth defects in both the wild-type and *mds3*Δ/Δ backgrounds (Fig. 5A and 6), demonstrating that the *TOR1-1* allele is not completely functional. Thus, we considered that this growth defect could influence our hyphal formation data. To address this, we constructed an *mds3*Δ/Δ *rbp1*Δ/Δ double mutant that did not have a growth defect (Fig. 5A). Rapamycin inhibits the TOR kinase as a complex with Rbp1 (FKBP12 in mammals), and *rbp1*Δ/Δ mutants are rapamycin resistant (Fig. 5A and reference 13). While *rbp1*Δ/Δ mutants did not have a defect in hyphal formation, the loss of Rbp1 in the *mds3*Δ/Δ background completely blocked the ability of rapamycin to rescue the *mds3*Δ/Δ hyphal formation defects (Fig. 5B and Table 6). The loss of Rbp1 also prevented the transcriptional effects of rapamycin in the *mds3*Δ/Δ mutant (Fig. 2A, lanes 16 to 18, and C, lanes 11 and 12). In total, these results clearly demonstrate that the transcriptional effects of the *mds3*Δ/Δ mutant have biological consequences that are associated with the inappropriate activation of the TOR pathway.

Mds3 and the TOR effector Sit4 act similarly. One downstream effector of the TOR pathway is the type 2A-like phosphatase Sit4 (22, 23, 66). Sit4 promotes starvation responses and is inhibited by TOR-dependent phosphorylation during periods of active growth (21, 40). Thus, in the absence of Sit4, TOR-dependent starvation responses are defective. Since the *mds3*Δ/Δ mutant also is defective for TOR-dependent starvation responses and promotes TOR activation (Table 4 and Fig. 2), we predicted that a *sit4*Δ/Δ mutant has transcriptional defects similar to those of the *mds3*Δ/Δ mutant. Indeed, in the

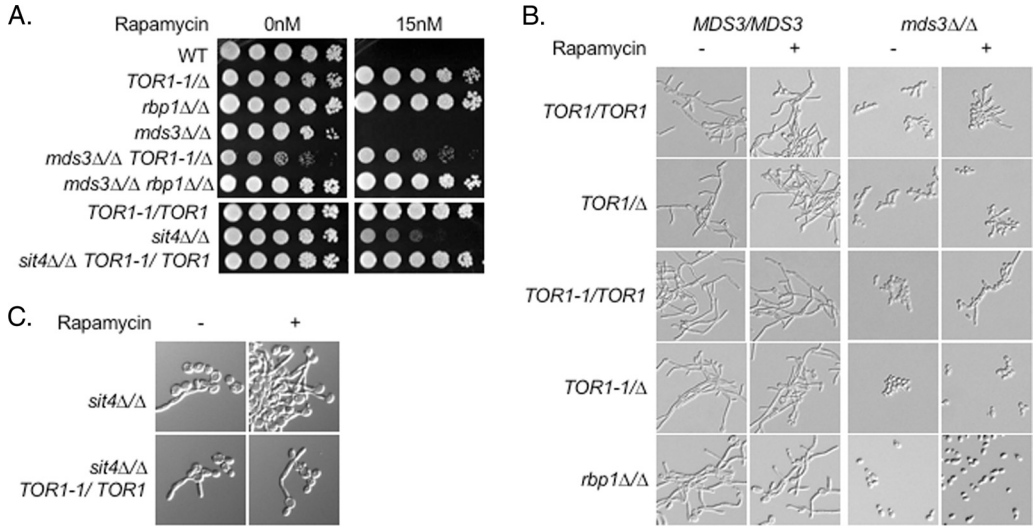

FIG. 5. Effect of rapamycin on filamentation is TOR dependent. (A) Wild-type (DAY185), *TOR1-1*/*tor1*Δ (DAY1125), *rbp1*Δ/Δ (DAY1223), *mds3*Δ/Δ (DAY1118), *mds3*Δ/Δ *TOR1-1*/*tor1*Δ (DAY1124), *mds3*Δ/Δ *rbp1*Δ/Δ (DAY1239), *TOR1-1*/*TOR1* (DAY1121), *sit4*Δ/Δ (DAY972), and *sit4*Δ/Δ *TOR1-1*/*TOR1* (DAY1321) strains were grown overnight in YPD, and serial dilutions were spotted onto YPD with or without 15 nM rapamycin. Overnight YPD cultures of the wild-type and the *mds3*Δ/Δ backgrounds carrying *TOR1*/*TOR1* (DAY185 and DAY1118), *TOR1*/Δ (DAY1123 and DAY1122), *TOR1-1*/*TOR1* (DAY1121 and DAY1255), and *TOR1-1*/Δ alleles (DAY1125 and DAY1124) or the *rbp1*Δ/Δ deletion (DAY1223 and DAY1239) (B) and *sit4*Δ/Δ *TOR1*/*TOR1* (DAY972) and *sit4*Δ/Δ *TOR1-1*/*TOR1* (DAY1321) (C) were washed in PBS and diluted 1:100 in M199 liquid medium (pH 8) with or without 5 nM rapamycin.

*sit4*Δ/Δ mutant, *CDC19*, *RPS26A*, and *TEF1* were expressed at higher levels than those of the wild-type strain at both pH 4 and 8 (Fig. 2A, compare lanes 4 and 9 to lanes 1 and 6) and in nutrient-poor Spider medium (Fig. 2B). We noted that at pH 4, *CDC19* expression in the *sit4*Δ/Δ mutant was ~4-fold higher than that in the *mds3*Δ/Δ mutant. Further, in the *sit4*Δ/Δ mutant, *GAP2* was not expressed at either pH 4 or 8, although it was expressed more in Spider medium, similarly to the *mds3*Δ/Δ mutant. Thus, the loss of Sit4 has transcriptional effects similar to those of the loss of Mds3.

The addition of rapamycin to *sit4*Δ/Δ cells reduced the expression of *CDC19*, *RPS26A*, and *TEF1* to approximately wild-type levels in M199 pH 8 medium (Fig. 2A, compare lanes 9 and 14) but had a more modest effect on Spider medium (Fig. 2B). Rapamycin also promoted the expression of *GAP2* in *sit4*Δ/Δ cells. However, in M199 pH 8 medium, *GAP2* expression still was ~2-fold lower than that of wild-type cells, indicating that Sit4 is required for the full induction of *GAP2*. These results demonstrate that the loss of Sit4 promotes the activation of the TOR pathway similarly to the loss of Mds3 and provides independent support for the idea that Mds3 acts in the TOR pathway.

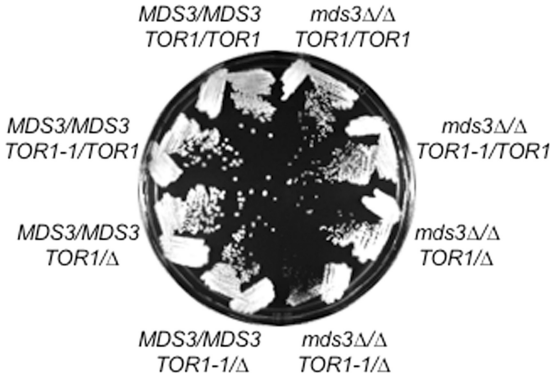

FIG. 6. *TOR1-1*/Δ *mds3*Δ/Δ mutant grows poorly. (A) Overnight YPD cultures of the wild-type and the *mds3*Δ/Δ backgrounds carrying *TOR1*/*TOR1* (DAY185 and DAY1118), *TOR1*/Δ (DAY1123 and DAY1122), *TOR1-1*/*TOR1* (DAY1121 and DAY1255), or *TOR1-1*/Δ alleles (DAY1125 and DAY1124) or the *rbp1*Δ/Δ deletion (DAY1223 and DAY1239) were streaked onto YPD medium at 30°C for 2 days.

Sit4 also is required for hyphal formation in *C. albicans* (46) (Fig. 3B). We found that *sit4*Δ/Δ mutant cells expressed little *HWP1* and *ECE1* in M199 pH 8 medium and that this defect was partially rescued by the addition of rapamycin (Fig. 3A, lanes 7 and 8). Similarly to the *mds3*Δ/Δ mutant, rapamycin rescued the *sit4*Δ/Δ hyphal formation defect in M199 pH 8 (12.9% ± 0.1% in solvent versus 60.5% ± 2.6% in rapamycin) and SLAD media (9.2% ± 1.0% in solvent versus 19.2% ± 0.5% in rapamycin) (Fig. 3B and 4A), and this rescue was prevented by the *TOR1-1* rapamycin-resistant allele (Fig. 5A and C). Importantly, rapamycin did not rescue the filamentation defects associated with the loss of Ras1 or Rim101, which are not associated with the TOR pathway (Fig. 3B). In total, these results suggest a functional association between Mds3 and Sit4.

TABLE 6. Hyphal formation in M199 pH 8 in the presence and absence of 5 nM rapamycin

Strain	Genotype	% of hyphae ± SEM	
		Solvent	Rapamycin
DAY185	*MDS3*/*MDS3 RBP1*/*RBP1*	75.6 ± 2.9	84.9 ± 4.0
DAY1233	*MDS3*/*MDS3 rbp1*Δ/*rbp1*Δ	91.4 ± 1.1	91.1 ± 1.7
DAY1118	*mds3*Δ/Δ *RBP1*/*RBP1*	4.4 ± 1.7	30.4 ± 3.5
DAY1239	*mds3*Δ/Δ *rbp1*Δ/*rbp1*Δ	3.0 ± 0.9	3.5 ± 1.0

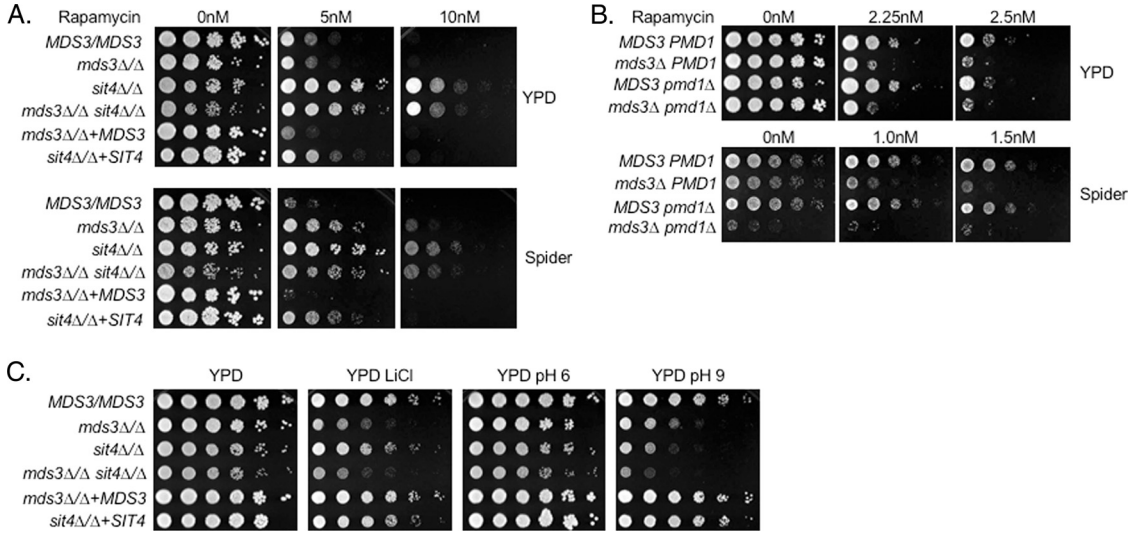

FIG. 7. Mds3 and TOR pathway members have altered rapamycin resistance. (A) *C. albicans* wild-type (DAY185), *mds3*Δ/Δ (DAY1118), *sit4*Δ/Δ (DAY972), *sit4*Δ/Δ +*SIT4* (DAY973), *mds3*Δ/Δ +*MDS3* (DAY1119), and *mds3*Δ/Δ *sit4*Δ/Δ (DAY1233) strains were serially diluted in PBS and spotted onto YPD or Spider media with or without rapamycin. (B) *S. cerevisiae* wild-type (DAY1208), *mds3*Δ *PMD1*(DAY1209), *MDS3 pmd1*Δ (DAY1210), and *mds3*Δ *pmd1*Δ (DAY1211) strains were serially diluted in PBS and spotted onto YPD or Spider media supplemented with or without rapamycin. Plates were photographed after 48 or 72 h of growth at 30°C. (C) The *C. albicans* strains listed for panel A were serially diluted in PBS and spotted onto YPD or YPD supplemented with 150 mM LiCl or were buffered at pH 6 or 9. Growth was scored after 48 h of incubation at 30°C.

To determine if Mds3 and Sit4 act in the same pathway or parallel pathways, we constructed an *mds3*Δ/Δ *sit4*Δ/Δ double mutant. If Mds3 and Sit4 function in the same pathway, then the *mds3*Δ/Δ *sit4*Δ/Δ double mutant should have phenotypes similar to those of either single mutant; if Mds3 and Sit4 function in parallel pathways, then the *mds3*Δ/Δ *sit4*Δ/Δ double mutant should have more severe phenotypes than either single mutant. The *mds3*Δ/Δ *sit4*Δ/Δ double mutant expressed *CDC19*, *RPS26A*, *TEF1*, and *GAP2* similarly to the *mds3*Δ/Δ and *sit4*Δ/Δ single mutants in M199 pH 8 and Spider media (Fig. 2A, compare lanes 7, 9, and 10). In M199 pH 4 medium, the *mds3*Δ/Δ *sit4*Δ/Δ double mutant behaved similarly to the *sit4*Δ/Δ single mutant, which had a more severe phenotype than the *mds3*Δ/Δ mutant (Fig. 2A, compare lanes 2, 4, and 5). In Spider medium, the *mds3*Δ/Δ *sit4*Δ/Δ double mutant also behaved similarly to the *sit4*Δ/Δ single mutant. However, the *mds3*Δ/Δ single mutant had a more severe phenotype, indicating a dominant effect of the *sit4*Δ/Δ mutation (Fig. 2B, compare lanes 2, 4, and 5). These results demonstrate that the *mds3*Δ/Δ *sit4*Δ/Δ mutant does not have transcriptional defects beyond that of the single mutants, suggesting Mds3 and Sit4 act in the same pathway. However, the addition of rapamycin did not restore *CDC19*, *RPS26A*, and *TEF1* expression in the *mds3*Δ/Δ *sit4*Δ/Δ double mutant (Fig. 2A, lanes 12, 14, and 15, and B, lanes 7, 9, and 10), although it did restore *GAP2* expression (Fig. 2A, lanes 10 and 15). Rapamycin also did not rescue the hyphal formation defect associated with the *mds3*Δ/Δ *sit4*Δ/Δ double mutant (Fig. 3 and 4A), which suggests that Mds3 and/or Sit4 has additional parallel functions.

We noted that regardless of pH, wild-type cells grown in M199 medium express relatively little *RPS26A* and robust levels of *GAP2*, suggesting that this medium is a nutrient-poor medium (Fig. 2A, lane 1). Since TOR responds to nutrient availability, we asked if Mds3 and Sit4 affect gene expression in a nutrient-rich environment. Thus, we determined *RPS26A* and *GAP2* expression in rich YPD medium with or without rapamycin. Unlike the results obtained for M199 pH 8 and Spider media (Fig. 2A and B), we observed the robust expression of *RPS26A* and no expression of *GAP2* in wild-type cells, and cells lacking Mds3 and/or Sit4 behaved similarly (Fig. 2C, compare lane 1 to lanes 2, 4, and 5). The addition of rapamycin reduced the expression of *RPS26A* and promoted the expression of *GAP2* in wild-type cells, as expected if TOR is inhibited (Fig. 2C, lane 6). The addition of rapamycin had a similar effect on *mds3*Δ/Δ, *sit4*Δ/Δ, and *mds3*Δ/Δ *sit4*Δ/Δ mutants (Fig. 2C, lanes 7, 9, and 10). Further, this effect is clearly due to TOR inhibition, as *RPS26A* and *GAP2* expression was similar in the *mds3*Δ/Δ *rbp1*Δ/Δ double mutant with and without rapamycin (Fig. 2C, lanes 11 and 12). We noted that *GAP2* expression in the presence of rapamycin was consistently higher in the *mds3*Δ/Δ mutant, and that this increase was *SIT4* dependent. This is in contrast to the results observed in M199 pH 8 medium, where *GAP2* expression in the *mds3*Δ/Δ mutant in the presence of rapamycin appears to be Sit4 independent. These results suggest that M199 medium is a more nutrient-poor medium than YPD and suggest that Mds3 and Sit4 contribute to the regulation of TOR-dependent targets in nutrient-poor conditions.

Mds3 promotes rapamycin sensitivity. Since rapamycin rescued the filamentation defects of the *mds3*Δ/Δ and the *sit4*Δ/Δ mutants, we wanted to determine if these mutations affected rapamycin sensitivity in *C. albicans*. On YPD medium, the wild-type, *mds3*Δ/Δ, and *mds3*Δ/Δ +*MDS3* strains showed similar rapamycin sensitivities, but the *sit4*Δ/Δ mutant was resistant to rapamycin (Fig. 7A). The complementation of the

*sit4*Δ/Δ mutant restored rapamycin sensitivity to wild-type levels. This result for the *sit4*Δ/Δ mutant is in sharp contrast to the situation in *S. cerevisiae*, where the *sit4*Δ mutant is sensitive to rapamycin and this sensitivity can be rescued by *C. albicans SIT4*, suggesting that Sit4 is functioning similarly in the two organisms (14, 46). These results indicate that *MDS3* does not affect rapamycin sensitivity in rich medium, and that unlike the case for *S. cerevisiae*, Sit4 function promotes rapamycin sensitivity in *C. albicans*.

Since Mds3 appears to promote TOR-dependent responses in nutrient-poor conditions, we considered that Mds3 affects rapamycin sensitivity on nutrient-poor medium. Indeed, while wild-type cells were sensitive to 5 nM rapamycin on nutrient-poor Spider medium, the *mds3*Δ/Δ mutant was resistant (Fig. 7A). Similar results were observed for the *sit4*Δ/Δ mutant. The *mds3*Δ/Δ *sit4*Δ/Δ double mutant behaved like the *sit4*Δ/Δ single mutant on both media, suggesting that Sit4 and Mds3 do not make independent contributions to rapamycin sensitivity. These results demonstrate that Mds3 and Sit4 promote rapamycin sensitivity in *C. albicans* and support the idea that different nutrient conditions have distinct constraints on the TOR pathway and the function of Mds3 and Sit4 in the TOR pathway.

Since the function of Sit4 in *S. cerevisiae* promotes rapamycin resistance (14), we predicted that the function of Mds3 in *S. cerevisiae* would be similar. To test this hypothesis, we determined the rapamycin sensitivity of congenic wild-type, *mds3*Δ, *pmd1*Δ (an *MDS3* paralog), and *mds3*Δ *pmd1*Δ *S. cerevisiae* strains (Fig. 7B). On rich medium, the wild type and *pmd1*Δ mutant grew similarly in the presence of rapamycin, and the *mds3*Δ and *mds3*Δ *pmd1*Δ mutants were more sensitive to rapamycin. Similar results were observed in nutrient-poor Spider medium; however, we noted that the *mds3*Δ *pmd1*Δ double mutant grew extremely poorly on this medium (Fig. 7B). Since the rapamycin sensitivities of the *mds3*Δ and *mds3*Δ *pmd1*Δ mutants were similar and since the *pmd1*Δ mutant did not confer rapamycin sensitivity, we conclude that Mds3, but not Pmd1, functions in the TOR pathway. Cells lacking Sit4 or Mds3 have similar phenotypes in relation to rapamycin sensitivity; however, these phenotypes are disparate between *S. cerevisiae* and *C. albicans*. Thus, we conclude that Mds3 and Sit4 function similarly in *C. albicans* and *S. cerevisiae* but that the effect of these functions has changed since *C. albicans* and *S. cerevisiae* split evolutionarily.

Because the *mds3*Δ/Δ and *sit4*Δ/Δ mutants share a number of phenotypic traits, we wanted to determine if the *sit4*Δ/Δ mutant had other *mds3*Δ/Δ-dependent phenotypes (18). While the *sit4*Δ/Δ mutant has a slight growth defect on YPD, YPD plus LiCl, or YPD pH 6 medium, the *sit4*Δ/Δ mutant had a severe growth defect on YPD pH 9 medium that was rescued by the reintroduction of a wild-type copy of *SIT4* (Fig. 7C). The *mds3*Δ/Δ *sit4*Δ/Δ double mutant grew more poorly on both YPD and YPD pH 9 medium than either single mutant (Fig. 7C), supporting the idea that Mds3 and Sit4 have some independent functions. The *mds3*Δ/Δ *sit4*Δ/Δ double mutant grew similarly to the *mds3*Δ/Δ mutant on LiCl medium, suggesting that Sit4 does not play a role in response to this stress medium. These results support a model in which Mds3 and Sit4 function similarly in the TOR pathway but are not completely dependent.

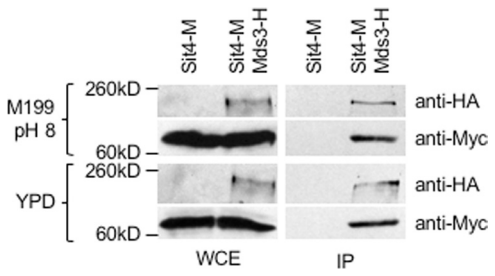

FIG. 8. Mds3 and Sit4 interact physically. *C. albicans* Sit4-Myc-tagged (Sit4-M) strains with and without Mds3-HA (Mds3-H) (DAY1236 and DAY1235, respectively) were grown in M199 (pH 8) and YPD. An aliquot of the whole-cell extracts (WCE) was separated by SDS-PAGE, and an aliquot was incubated with anti-HA beads, centrifuged, washed, and eluted (IP) prior to SDS-PAGE. Blots were probed with anti-HA first, stripped, and then probed with anti-Myc.

Mds3 and Sit4 physically interact. Mds3 was identified as a protein that interacts with Sit4 in an *S. cerevisiae* proteomic screen (27, 28). Since we have demonstrated a strong genetic interaction between Mds3 and Sit4, we tested whether Mds3 and Sit4 can indeed physically interact. Thus, we generated a strain carrying a functional C-terminal 13× Myc-tagged *SIT4* allele and then introduced a functional *MDS3* allele containing the 3× HA epitope. Whole-cell extracts of cells grown in M199 pH 8 medium were immunoprecipitated with α-HA and separated by SDS-PAGE. Sit4-Myc was detected in similar amounts in whole-cell extracts from strains with and without Mds3-HA (Fig. 8). In α-HA immunoprecipitates from cells containing Mds3-HA, Sit4-Myc was pulled down. Sit4-Myc was not detected in anti-HA immunoprecipitates in the absence of Mds3-HA, demonstrating that Sit4-Myc is associating specifically with Mds3-HA. Similar results were observed in whole-cell extracts from cells grown in YPD, indicating that this interaction is not dependent on nutrient availability (Fig. 8). Thus, Mds3 and Sit4 physically interact, demonstrating that Mds3 is a member of the TOR pathway.

DISCUSSION

Growth and morphogenesis is controlled by numerous signal transduction pathways, which respond to diverse environmental cues. The TOR pathway is a key regulator of growth and morphogenesis in eukaryotes, and in *C. albicans*, the TOR pathway governs the yeast-to-hyphal morphogenetic transition, which is critical for pathogenesis (14, 77). Microarray analyses suggested that *MDS3* is a member of the TOR pathway in *C. albicans*, and this idea was supported by genetic, pharmacological, and biochemical approaches. Thus, Mds3 represents a new member of the TOR morphogenetic pathway.

How does Mds3 contribute to the TOR pathway? Mds3 is a 1,383-amino-acid protein with predicted Kelch repeats in the N-terminal region but no other obvious motifs and functional domains (18). Kelch repeats form a β-propeller structure that functions as a protein-protein interaction domain and is found in an array of proteins, including cytoskeletal proteins and signal transduction proteins (2, 25, 34, 50). To the best of our knowledge, Mds3 represents the first Kelch repeat protein associated with the TOR pathway. However, two components of the TOR complex, Lst8 and Kog1 (GβL and Raptor in

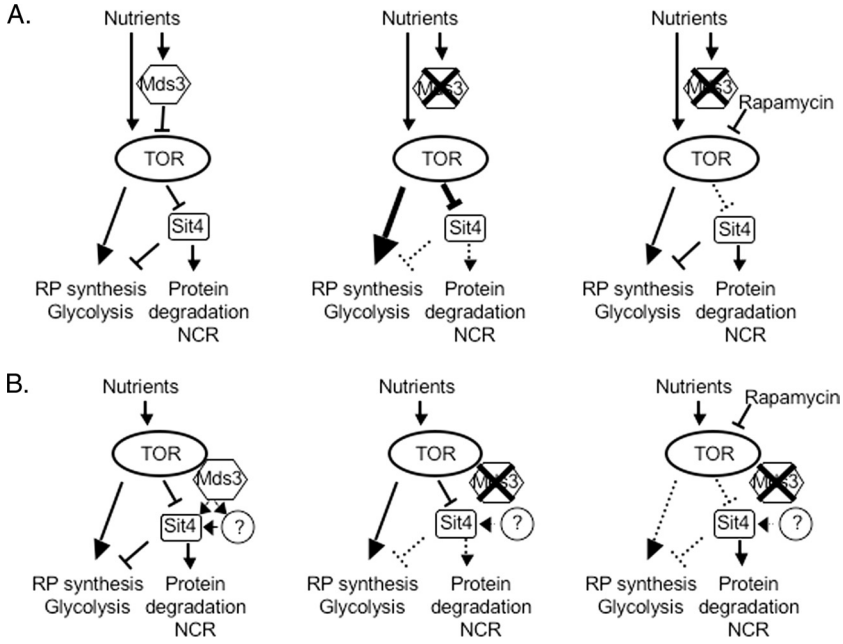

FIG. 9. Models of Mds3 as a TOR pathway member. (A) Mds3 as an upstream negative regulator of TOR (left). The loss of Mds3 leads to the increased the expression of genes involved in translation and the increased repression of Sit4 and less expression of the NCR genes (middle). This loss of negative regulation can be restored with rapamycin (right). (B) Mds3 as a downstream member of the Sit4-dependent pathway inhibited by TOR. This Sit4-dependent pathway inhibits the expression of RP synthesis genes and promotes the expression of NCR genes (left). The loss of Mds3 (or Sit4) leads to the increased expression of genes involved in translation and the decreased expression of the NCR genes (middle). Rapamycin inhibits TOR activation, restoring RP synthesis expression by reduced TOR kinase function. NCR expression also is restored, although this mechanism is not yet understood (right).

mammalian systems), contain WD-40 domains, which also fold into β-propeller structures (9, 31, 41, 42, 49). Thus, we propose that Mds3 acts as a scaffold to facilitate interactions between TOR pathway members to control morphogenetic processes in *C. albicans*.

Based on our results, which include the transcriptional and morphological similarities between the *mds3*Δ/Δ and *sit4*Δ/Δ mutants that are rescued by rapamycin and the interaction between Mds3 and Sit4, we propose two models to explain how Mds3 acts in the TOR pathway (Fig. 9). First, Mds3 may act upstream of TOR as a member of a nutrient-sensitive complex, such as the one composed of Raptor, GβL, and mTOR (41, 42). Support for this model comes from the fact that rapamycin, which inhibits TOR kinase activity, rescues the transcriptional defects and morphogenetic defects associated with the *mds3*Δ/Δ mutant (Fig. 2 to 4). Further, the rapamycin-mediated rescue of the *mds3*Δ/Δ mutant is lost with the introduction of a *TOR1-1* allele or the loss of Rbp1 (Fig. 2 and 5). Based on these data, Mds3 appears to act upstream of TOR. However, for this model to be accurate, Mds3 interacts indirectly with Sit4.

How could Mds3 function upstream of TOR? One possibility is that Mds3 modulates the interaction of Kog1 and Lst8, Raptor and GβL in mammalian systems, with TOR. GβL promotes TOR kinase activity, and Raptor-GβL constitutively interacts with mTOR. However, Raptor-GβL-mTOR interaction is affected by nutrient signals (41). Under nutrient-poor conditions Raptor-mTOR interaction is stabilized, which interferes with the GβL stimulation of mTOR (42). In yeast, Kog1-Lst8 interaction with Tor1 does not appear to be affected by nutrient status (49). This led to the hypothesis that an additional factor, such as Mds3, is involved that affects the activity of Kog1 and/or Lst8 on Tor1.

An alternative model is that Mds3 acts downstream of Tor1 to promote starvation responses when Tor1 is inactive (Fig. 9). Support for this model comes from the fact that the *sit4*Δ/Δ mutant has transcriptional and morphological defects similar to those of the *mds3*Δ/Δ mutant, and that these defects are rescued by rapamycin (Fig. 2 to 5). Additionally, both *mds3*Δ/Δ and *sit4*Δ/Δ mutants are sensitive to alkaline pH (Fig. 7C). Since rapamycin inhibits TOR kinase activity, these results suggest that the transcriptional and morphogenetic defects associated with the *mds3*Δ/Δ and *sit4*Δ/Δ mutants are due to an increase in TOR kinase activity or TOR kinase-dependent activities, and that reduced kinase activity by rapamycin is sufficient to rescue the *sit4*Δ/Δ and *mds3*Δ/Δ mutants. Finally, the fact that Mds3 and Sit4 coimmunoprecipitate suggests that these proteins interact within the same complex either directly or indirectly. One potential problem for this model is the restoration of *GAP2* expression by rapamycin in the *mds3*Δ/Δ mutant (Fig. 2). However, *GAP2* expression also is rescued by rapamycin in the *sit4*Δ/Δ mutant. Thus, additional PP2As, which are activated by rapamycin treatment, may promote the expression of *GAP2* independently of Mds3 or Sit4. A similar phenomenon has been described for *S. cerevisiae* (11, 29, 69). Finally, we noted that the *TOR1-1* allele and *mds3*Δ/Δ mutation had a synergistic effect, which generally suggests parallel pathways. However, the *TOR1-1* allele and similar alleles have altered functions in both *S. pombe* and mammalian systems (55, 73, 75), and we find that *TOR1-1* confers growth defects in

the absence of a wild-type *TOR1* allele (Fig. 5A and 6B). Thus, we propose that the *TOR1-1 mds3*Δ/Δ synergism reflects defective kinase function in Tor1-1, which exacerbates the TOR pathway defects associated with the *mds3*Δ/Δ mutant.

How could Mds3 function downstream of TOR? One possibility is that Mds3 acts as a scaffold bridging TOR activity to its effectors. For example, Sit4 activity is dependent upon its association with Tap42 and several Sit4-associated proteins (SAPs), and Mds3 may govern these associations (20, 22, 51). Mds3 may facilitate or stabilize these interactions to promote Sit4 activity. Support for this idea comes from large-scale protein-protein interaction analyses that identified Sap185, a Sit4 regulatory protein, as an Mds3 binding partner (20, 27, 28, 51).

Mds3, TOR, and nutrient sensing. TOR kinase activity is controlled by nutrient availability (12, 20, 41, 66). In nutrient-rich conditions, TOR kinase activity is stimulated, leading to the activation of growth processes and the inactivation of starvation responses. In starvation conditions, TOR kinase activity is inhibited, preventing the activation of growth responses and leading to the activation of starvation responses. Here, we found that wild-type cells promote the expression of *RPS26A* and repression of *GAP2* when grown in YPD but promote the expression of *RPS26A*, although to reduced levels compared to those for YPD, and *GAP2* when grown in M199 (Fig. 2). This suggests that M199 is nutrient poor compared to YPD. Further, wild-type cells divide slower in M199 pH 8 medium than in M199 pH 4 medium, suggesting that M199 pH 8 is more poor than M199 pH 4 medium. The latter growth difference can be explained by the inhibition of plasma membrane transporters and the dependence on the endocytic uptake of nutrients in alkali environments (57, 71, 76). Thus, YPD > M199 pH 4 > M199 pH 8 in nutrient availability, and cells grown in these media may require different levels of TOR kinase activity.

We found that the *mds3*Δ/Δ and *sit4*Δ/Δ mutants did not affect the expression of TOR-dependent genes when grown in YPD, as expected if Mds3 and Sit4 promote TOR-dependent starvation responses (Fig. 2). In M199 pH 8 medium and Spider medium, the *mds3*Δ/Δ and *sit4*Δ/Δ mutants had similar defects, suggesting that both media are nutrient poor and require TOR-dependent starvation responses. In M199 pH 4 medium, the *mds3*Δ/Δ and *sit4*Δ/Δ mutants did not repress *CDC19*, *RPS26A*, or *TEF1*, nor did they induce *GAP2* to wild-type levels, supporting the idea that M199 pH 4 medium is a semistarvation medium that requires some level of the TOR-dependent starvation response. In M199 pH 4 medium, the *sit4*Δ/Δ mutant clearly had a more severe defect in the repression of *CDC19* and *RPS26A* and induction of *GAP2* than the *mds3*Δ/Δ mutant. This represents the first demonstration that Sit4 negatively regulates RP synthesis and is in contrast to work with *S. cerevisiae*, which suggests that Sit4 does not govern RP synthesis (23, 67). However, these results suggest that Sit4 plays a more general role in promoting TOR-dependent starvation responses than Mds3. For example, Sit4 may be required under both semistarvation and starvation conditions, whereas Mds3 may function only under starvation conditions. This idea is supported by the fact that the *mds3*Δ/Δ mutant was resistant to rapamycin in nutrient-poor medium but not in rich medium, whereas the *sit4*Δ/Δ mutant was resistant to rapamycin in both media (Fig. 7A).

Mds3 function in *C. albicans* and *S. cerevisiae*. *MDS3* was first identified in *S. cerevisiae* as a multicopy suppressor of the Δ*mck1* sporulation defect and subsequently was identified as a regulator of the *C. albicans* yeast-to-hypha transition (5, 18). These morphogenetic processes are promoted by neutral-alkaline environmental pH, which led to the idea that Mds3 is a pH response regulator that is similar to Rim101. This idea was supported by the fact that Mds3 is required for wild-type growth at alkaline pH (18). However, Mds3 also is required for chlamydospore and biofilm formation in *C. albicans*, neither of which require neutral-alkaline pH (60, 65). We also found that the *mds3*Δ/Δ mutant has a slight growth defect on rich medium, which is acidic, and our microarray studies demonstrated that the *mds3*Δ/Δ mutant showed transcriptional changes at both acidic and alkaline pH (Fig. 1 to 3A and 6 and Tables 3 and 4; also see Table S1 in the supplemental material). Thus, Mds3 clearly plays a broader role than neutral-alkaline pH regulation in *C. albicans*, and we propose that the morphogenetic defects observed in the *mds3*Δ/Δ mutant reflect defects in general cellular processes that also are required for growth and filamentation at alkaline pH.

In *S. cerevisiae*, Mds3 and Pmd1 are proposed to act upstream of or in parallel to the Ras/PKA pathway (5, 54), because the sporulation defect of the *mds3*Δ *pmd1*Δ double mutant was rescued by the hyperactive $RAS2^{V19}$ allele (5). However, several lines of evidence suggest that Mds3 is unlikely to function upstream of Ras independently of TOR. First, the hyperactive $RAS2^{V19}$ allele rescues TOR pathway mutants in *S. cerevisiae*, suggesting that Ras2 acts in parallel or downstream of the TOR pathway (14, 67, 81). Second, the filamentation defect of the *ras1*Δ/Δ mutant is not rescued by rapamycin, unlike the *mds3*Δ/Δ mutant (Fig. 7 and reference 26, 45). Third, we found that both the Δ*sit4* and Δ*mds3* mutants are sensitive to rapamycin in *S. cerevisiae*. Thus, we propose that Mds3 acts in the TOR pathway in both *S. cerevisiae* and *C. albicans*, but that the hyperactive $RAS2^{V19}$ allele is able to bypass this by acting either in parallel or downstream of TOR.

Why does *S. cerevisiae* encode two *MDS3* paralogs? In *S. cerevisiae*, phenotypes associated with the Δ*mds3* mutant are masked by the functionally redundant *PMD1* (5, 18). However, we found that the *S. cerevisiae* Δ*mds3* mutant, like the Δ*sit4* mutant but not the Δ*pmd1* mutant, was sensitive to rapamycin, which is the first demonstration that Mds3 and Pmd1 are not completely redundant in *S. cerevisiae*. In *C. albicans*, the *mds3*Δ/Δ and *sit4*Δ/Δ mutants behave similarly; however, our results suggest that Mds3 has Sit4-independent functions. First, the *mds3*Δ/Δ mutant is sensitive to high concentrations of LiCl, whereas the *sit4*Δ/Δ mutant is not (Fig. 7C and reference 18). Second, the *mds3*Δ/Δ *sit4*Δ/Δ double mutant has more severe growth defects on YPD and at alkaline pH than either single mutant (Fig. 7C). Third, the filamentation defect of the *mds3*Δ/Δ *sit4*Δ/Δ double mutant is not rescued by rapamycin (Fig. 3 and 4). Thus, in *C. albicans*, Mds3 has Sit4-dependent and -independent function. This raises the interesting possibility that in *S. cerevisiae*, Mds3 governs the Sit4-dependent functions, rapamycin sensitivity, and Pmd1 governs the Sit4-independent functions. It is not yet known if Sit4-independent functions of Mds3 in *C. albicans* occur through the TOR pathway or completely independent of the TOR pathway.

While Mds3 appears to act in the TOR pathway in both *C.*

albicans and *S. cerevisiae*, our results have demonstrated that the roles of the TOR pathway in these organisms have diverged. In *S. cerevisiae*, sporulation requires both nitrogen and carbon starvation and is negatively regulated by Mds3. This suggests that Mds3 functions as a positive regulator of the TOR pathway (5, 54). However, we have clearly shown that Mds3 functions as a negative regulator of the TOR pathway in *C. albicans* during starvation conditions. Thus, while the TOR pathway is mechanistically similar in *C. albicans* and *S. cerevisiae*, the signals coming in to and/or responses coming out of the TOR pathway appear to be distinct. This idea is supported by the rapamycin resistance observed for the *mds3*Δ/Δ and *sit4*Δ/Δ mutants in *C. albicans* compared to the sensitivity observed for these mutants in *S. cerevisiae* (Fig. 7B and reference 14, 46). Regardless, several signal transduction pathways have been identified that, while mechanistically similar, lead to distinct responses in *C. albicans* and *S. cerevisiae*, which is not surprising given the ~300 million years of evolutionary distance between these organisms (37, 39, 52, 70).

Despite the fact that rapamycin initially triggered interest because of its antifungal properties against *C. albicans* (72), the TOR pathway has received relatively little attention in this organism. In fact, most work has focused on the role of TOR in multicellular organisms in processes as diverse as angiogenesis and embryogenesis. However, TOR appears to have conserved functions in growth and stress responses in both multicellular and unicellular eukaryotes (64, 79). Our studies demonstrate that the kelch repeat protein Mds3, which promotes morphogenetic processes critical for pathogenesis, such as the yeast-to-hypha transition and biofilm formation, is a new member of the TOR pathway (15, 44, 59). Our study represents the first example in which a kelch repeat protein has been implicated in the TOR pathway and raises the possibility that an Mds3-like protein exists in mammalian systems to promote TOR-dependent responses.

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REFERENCES

1. **Adams, A., D. E. Gotschling, C. A. Kaiser, and T. Stearns.** 1997. Methods in yeast genetics, 1997 ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.
2. **Adams, J., R. Kelso, and L. Cooley.** 2000. The kelch repeat superfamily of proteins: propellers of cell function. Trends Cell Biol. **10:**17–24.
3. **Alonso-Monge, R., E. Roman, D. M. Arana, J. Pla, and C. Nombela.** 2009. Fungi sensing environmental stress. Clin. Microbiol. Infect. **15**(Suppl. 1)**:**17–19.
4. **Bastidas, R. J., J. Heitman, and M. E. Cardenas.** 2009. The protein kinase Tor1 regulates adhesin gene expression in Candida albicans. PLoS Pathog. **5:**e1000294.
5. **Benni, M. L., and L. Neigeborn.** 1997. Identification of a new class of negative regulators affecting sporulation-specific gene expression in yeast. Genetics **147:**1351–1366.
6. **Bensen, E. S., S. J. Martin, M. Li, J. Berman, and D. A. Davis.** 2004. Transcriptional profiling in C. albicans reveals new adaptive responses to extracellular pH and functions for Rim101p. Mol. Microbiol. **54:**1335–1351.
7. **Cafferkey, R., P. R. Young, M. M. McLaughlin, D. J. Bergsma, Y. Koltin, G. M. Sathe, L. Faucette, W. K. Eng, R. K. Johnson, and G. P. Livi.** 1993. Dominant missense mutations in a novel yeast protein related to mammalian phosphatidylinositol 3-kinase and VPS34 abrogate rapamycin cytotoxicity. Mol. Cell. Biol. **13:**6012–6023.
8. **Cardenas, M. E., N. S. Cutler, M. C. Lorenz, C. J. Di Como, and J. Heitman.** 1999. The TOR signaling cascade regulates gene expression in response to nutrients. Genes Dev. **13:**3271–3279.
9. **Chen, E. J., and C. A. Kaiser.** 2003. LST8 negatively regulates amino acid biosynthesis as a component of the TOR pathway. J. Cell Biol. **161:**333–347.
10. **Cottier, F., and F. A. Muhlschlegel.** 2009. Sensing the environment: response of Candida albicans to the X factor. FEMS Microbiol. Lett. **295:**1–9.
11. **Cox, K. H., A. Kulkarni, J. J. Tate, and T. G. Cooper.** 2004. Gln3 phosphorylation and intracellular localization in nutrient limitation and starvation differ from those generated by rapamycin inhibition of Tor1/2 in Saccharomyces cerevisiae. J. Biol. Chem. **279:**10270–10278.
12. **Crespo, J. L., T. Powers, B. Fowler, and M. N. Hall.** 2002. The TOR-controlled transcription activators GLN3, RTG1, and RTG3 are regulated in response to intracellular levels of glutamine. Proc. Natl. Acad. Sci. U. S. A. **99:**6784–6789.
13. **Cruz, M. C., A. L. Goldstein, J. Blankenship, M. Del Poeta, J. R. Perfect, J. H. McCusker, Y. L. Bennani, M. E. Cardenas, and J. Heitman.** 2001. Rapamycin and less immunosuppressive analogs are toxic to Candida albicans and Cryptococcus neoformans via FKBP12-dependent inhibition of TOR. Antimicrob. Agents Chemother. **45:**3162–3170.
14. **Cutler, N. S., X. Pan, J. Heitman, and M. E. Cardenas.** 2001. The TOR signal transduction cascade controls cellular differentiation in response to nutrients. Mol. Biol. Cell **12:**4103–4113.
15. **Davis, D., J. E. Edwards, Jr., A. P. Mitchell, and A. S. Ibrahim.** 2000. *Candida albicans RIM101* pH response pathway is required for host-pathogen interactions. Infect. Immun. **68:**5953–5959.
16. **Davis, D., R. B. Wilson, and A. P. Mitchell.** 2000. *RIM101*-dependent and-independent pathways govern pH responses in *Candida albicans*. Mol. Cell. Biol. **20:**971–978.
17. **Davis, D. A.** 2009. How human pathogenic fungi sense and adapt to pH: the link to virulence. Curr. Opin. Microbiol. **12:**365–370.
18. **Davis, D. A., V. M. Bruno, L. Loza, S. G. Filler, and A. P. Mitchell.** 2002. *Candida albicans* Mds3p, a conserved regulator of pH responses and virulence identified through insertional mutagenesis. Genetics **162:**1573–1581.
19. **Delgoffe, G. M., T. P. Kole, Y. Zheng, P. E. Zarek, K. L. Matthews, B. Xiao, P. F. Worley, S. C. Kozma, and J. D. Powell.** 2009. The mTOR kinase differentially regulates effector and regulatory T cell lineage commitment. Immunity **30:**832–844.
20. **Di Como, C. J., and K. T. Arndt.** 1996. Nutrients, via the Tor proteins, stimulate the association of Tap42 with type 2A phosphatases. Genes Dev. **10:**1904–1916.
21. **Di Como, C. J., and Y. Jiang.** 2006. The association of Tap42 phosphatase complexes with TORC1: another level of regulation in Tor signaling. Cell Cycle **5:**2729–2732.
22. **Düvel, K., and J. R. Broach.** 2004. The role of phosphatases in TOR signaling in yeast. Curr. Top. Microbiol. Immunol. **279:**19–38.
23. **Düvel, K., A. Santhanam, S. Garrett, L. Schneper, and J. R. Broach.** 2003. Multiple roles of Tap42 in mediating rapamycin-induced transcriptional changes in yeast. Mol. Cell **11:**1467–1478.
24. **Esposito, R. E., and S. Klapholz.** 1981. Meiosis and ascospore development, p. 211–288. *In* J. N. Strathern, E. W. Jones, and J. R. Broach (ed.), The molecular biology of the yeast Saccharomyces: life cycle and inheritance, 11A ed. Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.
25. **Feierbach, B., F. Verde, and F. Chang.** 2004. Regulation of a formin complex by the microtubule plus end protein tea1p. J. Cell Biol. **165:**697–707.
26. **Feng, Q., E. Summers, B. Guo, and G. Fink.** 1999. Ras signaling is required for serum-induced hyphal differentiation in Candida albicans. J. Bacteriol. **181:**6339–6346.
27. **Gavin, A. C., P. Aloy, P. Grandi, R. Krause, M. Boesche, M. Marzioch, C. Rau, L. J. Jensen, S. Bastuck, B. Dumpelfeld, A. Edelmann, M. A. Heurtier, V. Hoffman, C. Hoefert, K. Klein, M. Hudak, A. M. Michon, M. Schelder, M. Schirle, M. Remor, T. Rudi, S. Hooper, A. Bauer, T. Bouwmeester, G. Casari, G. Drewes, G. Neubauer, J. M. Rick, B. Kuster, P. Bork, R. B. Russell, and G. Superti-Furga.** 2006. Proteome survey reveals modularity of the yeast cell machinery. Nature **440:**631–636.
28. **Gavin, A. C., M. Bosche, R. Krause, P. Grandi, M. Marzioch, A. Bauer, J. Schultz, J. M. Rick, A. M. Michon, C. M. Cruciat, M. Remor, C. Hofert, M. Schelder, M. Brajenovic, H. Ruffner, A. Merino, K. Klein, M. Hudak, D. Dickson, T. Rudi, V. Gnau, A. Bauch, S. Bastuck, B. Huhse, C. Leutwein, M. A. Heurtier, R. R. Copley, A. Edelmann, E. Querfurth, V. Rybin, G. Drewes, M. Raida, T. Bouwmeester, P. Bork, B. Seraphin, B. Kuster, G. Neubauer, and G. Superti-Furga.** 2002. Functional organization of the yeast proteome by systematic analysis of protein complexes. Nature **415:**141–147.
29. **Georis, I., A. Feller, J. J. Tate, T. G. Cooper, and E. Dubois.** 2009. Nitrogen catabolite repression-sensitive transcription as a readout of Tor pathway regulation: the genetic background, reporter gene and GATA factor assayed determine the outcomes. Genetics **181:**861–874.

30. **Gerami-Nejad, M., K. Dulmage, and J. Berman.** 2009. Additional cassettes for epitope and fluorescent fusion proteins in Candida albicans. Yeast **26:** 399–406.
31. **Gettemans, J., K. Meerschaert, J. Vandekerckhove, and V. De Corte.** 2003. A kelch beta propeller featuring as a G beta structural mimic: reinventing the wheel? Sci. STKE **2003:**PE27.
32. **Gow, N. A., A. J. Brown, and F. C. Odds.** 2002. Fungal morphogenesis and host invasion. Curr. Opin. Microbiol. **5:**366–371.
33. **Han, J., B. Wang, Z. Xiao, Y. Gao, Y. Zhao, J. Zhang, B. Chen, X. Wang, and J. Dai.** 2008. Mammalian target of rapamycin (mTOR) is involved in the neuronal differentiation of neural progenitors induced by insulin. Mol. Cell Neurosci. **39:**118–124.
34. **Harashima, T., and J. Heitman.** 2002. The Galpha protein Gpa2 controls yeast differentiation by interacting with kelch repeat proteins that mimic Gbeta subunits. Mol. Cell **10:**163–173.
35. **Hardwick, J. S., F. G. Kuruvilla, J. K. Tong, A. F. Shamji, and S. L. Schreiber.** 1999. Rapamycin-modulated transcription defines the subset of nutrient-sensitive signaling pathways directly controlled by the Tor proteins. Proc. Natl. Acad. Sci. U. S. A. **96:**14866–14870.
36. **Heitman, J., N. R. Movva, and M. N. Hall.** 1991. Targets for cell cycle arrest by the immunosuppressant rapamycin in yeast. Science **253:**905–909.
37. **Hogues, H., H. Lavoie, A. Sellam, M. Mangos, T. Roemer, E. Purisima, A. Nantel, and M. Whiteway.** 2008. Transcription factor substitution during the evolution of fungal ribosome regulation. Mol. Cell **29:**552–562.
38. **Hwang, M., C. A. Perez, L. Moretti, and B. Lu.** 2008. The mTOR signaling network: insights from its role during embryonic development. Curr. Med. Chem. **15:**1192–1208.
39. **Ihmels, J., S. Bergmann, M. Gerami-Nejad, I. Yanai, M. McClellan, J. Berman, and N. Barkai.** 2005. Rewiring of the yeast transcriptional network through the evolution of motif usage. Science **309:**938–940.
40. **Jiang, Y., and J. R. Broach.** 1999. Tor proteins and protein phosphatase 2A reciprocally regulate Tap42 in controlling cell growth in yeast. EMBO J. **18:**2782–2792.
41. **Kim, D.-H., D. D. Sarbassov, S. M. Ali, J. E. King, R. R. Latek, H. Erdjument-Bromage, P. Tempst, and D. M. Sabatini.** 2002. mTOR interacts with raptor to form a nutrient-sensitive complex that signals to the cell growth machinery. Cell **110:**163–175.
42. **Kim, D. H., D. D. Sarbassov, S. M. Ali, R. R. Latek, K. V. Guntur, H. Erdjument-Bromage, P. Tempst, and D. M. Sabatini.** 2003. GbetaL, a positive regulator of the rapamycin-sensitive pathway required for the nutrient-sensitive interaction between raptor and mTOR. Mol. Cell **11:**895–904.
43. **Kullas, A. L., S. J. Martin, and D. Davis.** 2007. Adaptation to environmental pH: integrating the Rim101 and calcineurin signal transduction pathways. Mol. Microbiol. **66:**858–871.
44. **Kumamoto, C. A., and M. D. Vinces.** 2005. Contributions of hyphae and hypha-co-regulated genes to Candida albicans virulence. Cell Microbiol. **7:**1546–1554.
45. **Leberer, E., D. Harcus, D. Dignard, L. Johnson, S. Ushinsky, D. Y. Thomas, and K. Schroppel.** 2001. Ras links cellular morphogenesis to virulence by regulation of the MAP kinase and cAMP signalling pathways in the pathogenic fungus Candida albicans. Mol. Microbiol. **42:**673–687.
46. **Lee, C. M., A. Nantel, L. Jiang, M. Whiteway, and S. H. Shen.** 2004. The serine/threonine protein phosphatase SIT4 modulates yeast-to-hypha morphogenesis and virulence in Candida albicans. Mol. Microbiol. **51:**691–709.
47. **Liu, H., J. Kohler, and G. R. Fink.** 1994. Suppression of hyphal formation in *Candida albicans* by mutation of a *STE12* homolog. Science **266:**1723–1726.
48. **Lo, H. J., J. R. Kohler, B. DiDomenico, D. Loebenberg, A. Cacciapuoti, and G. R. Fink.** 1997. Nonfilamentous *C. albicans* mutants are avirulent. Cell **90:**939–949.
49. **Loewith, R., E. Jacinto, S. Wullschleger, A. Lorberg, J. L. Crespo, D. Bonenfant, W. Oppliger, P. Jenoe, and M. N. Hall.** 2002. Two TOR complexes, only one of which is rapamycin sensitive, have distinct roles in cell growth control. Mol. Cell **10:**457–468.
50. **Lu, A., and J. P. Hirsch.** 2005. Cyclic AMP-independent regulation of protein kinase A substrate phosphorylation by Kelch repeat proteins. Eukaryot. Cell **4:**1794–1800.
51. **Luke, M. M., F. Della Seta, C. J. Di Como, H. Sugimoto, R. Kobayashi, and K. T. Arndt.** 1996. The SAP, a new family of proteins, associate and function positively with the SIT4 phosphatase. Mol. Cell. Biol. **16:**2744–2755.
52. **Martchenko, M., A. Levitin, H. Hogues, A. Nantel, and M. Whiteway.** 2007. Transcriptional rewiring of fungal galactose-metabolism circuitry. Curr. Biol. **17:**1007–1013.
53. **Martin, K. A., E. M. Rzucidlo, B. L. Merenick, D. C. Fingar, D. J. Brown, R. J. Wagner, and R. J. Powell.** 2004. The mTOR/p70 S6K1 pathway regulates vascular smooth muscle cell differentiation. Am. J. Physiol. Cell Physiol. **286:**C507–C517.
54. **McDonald, C. M., M. Wagner, M. J. Dunham, M. E. Shin, N. T. Ahmed, and E. Winter.** 2009. The Ras/cAMP pathway and the CDK-like kinase Ime2 regulate the MAPK Smk1 and spore morphogenesis in Saccharomyces cerevisiae. Genetics **181:**511–523.
55. **McMahon, L. P., K. M. Choi, T. A. Lin, R. T. Abraham, and J. C. Lawrence, Jr.** 2002. The rapamycin-binding domain governs substrate selectivity by the mammalian target of rapamycin. Mol. Cell. Biol. **22:**7428–7438.
56. **Mitchell, A. P.** 1998. Dimorphism and virulence in Candida albicans. Curr. Opin. Microbiol. **1:**687–692.
57. **Munn, A. L., and H. Riezman.** 1994. Endocytosis is required for the growth of vacuolar H(+)-ATPase-defective yeast: identification of six new *END* genes. J. Cell Biol. **127:**373–386.
58. **Murad, A. M., P. Leng, M. Straffon, J. Wishart, S. Macaskill, D. MacCallum, N. Schnell, D. Talibi, D. Marechal, F. Tekaia, C. d'Enfert, C. Gaillardin, F. C. Odds, and A. J. Brown.** 2001. *NRG1* represses yeast-hypha morphogenesis and hypha-specific gene expression in *Candida albicans*. EMBO J. **20:**4742–4752.
59. **Nobile, C. J., D. R. Andes, J. E. Nett, F. J. Smith, F. Yue, Q. T. Phan, J. E. Edwards, S. G. Filler, and A. P. Mitchell.** 2006. Critical role of Bcr1-dependent adhesins in C. albicans biofilm formation in vitro and in vivo. PLoS Pathog. **2:**e63.
60. **Nobile, C. J., V. M. Bruno, M. L. Richard, D. A. Davis, and A. P. Mitchell.** 2003. Genetic control of chlamydospore formation in Candida albicans. Microbiology **149:**3629–3637.
61. **Nobile, C. J., N. Solis, C. L. Myers, A. J. Fay, J. S. Deneault, A. Nantel, A. P. Mitchell, and S. G. Filler.** 2008. Candida albicans transcription factor Rim101 mediates pathogenic interactions through cell wall functions. Cell Microbiol. **10:**2180–2196.
62. **Porta, A., A. M. Ramon, and W. A. Fonzi.** 1999. *PRR1*, a homolog of *Aspergillus nidulans palF*, controls pH-dependent gene expression and filamentation in *Candida albicans*. J. Bacteriol. **181:**7516–7523.
63. **Ramon, A. M., A. Porta, and W. A. Fonzi.** 1999. Effect of environmental pH on morphological development of *Candida albicans* is mediated via the PacC-related transcription factor encoded by *PRR2*. J. Bacteriol. **181:**7524–7530.
64. **Reiling, J. H., and D. M. Sabatini.** 2006. Stress and mTORture signaling. Oncogene **25:**6373–6383.
65. **Richard, M. L., C. J. Nobile, V. M. Bruno, and A. P. Mitchell.** 2005. Candida albicans biofilm-defective mutants. Eukaryot. Cell **4:**1493–1502.
66. **Rohde, J. R., and M. E. Cardenas.** 2004. Nutrient signaling through TOR kinases controls gene expression and cellular differentiation in fungi. Curr. Top. Microbiol. Immunol. **279:**53–72.
67. **Schmelzle, T., T. Beck, D. E. Martin, and M. N. Hall.** 2004. Activation of the RAS/cyclic AMP pathway suppresses a TOR deficiency in yeast. Mol. Cell. Biol. **24:**338–351.
68. **Shamji, A. F., F. G. Kuruvilla, and S. L. Schreiber.** 2000. Partitioning the transcriptional program induced by rapamycin among the effectors of the Tor proteins. Curr. Biol. **10:**1574–1581.
69. **Tate, J. J., I. Georis, A. Feller, E. Dubois, and T. G. Cooper.** 2009. Rapamycin-induced Gln3 dephosphorylation is insufficient for nuclear localization: Sit4 and PP2A phosphatases are regulated and function differently. J. Biol. Chem. **284:**2522–2534.
70. **Tsong, A. E., B. B. Tuch, H. Li, and A. D. Johnson.** 2006. Evolution of alternative transcriptional circuits with identical logic. Nature **443:**415–420.
71. **van der Rest, M. E., A. H. Kamminga, A. Nakano, Y. Anraku, B. Poolman, and W. N. Konings.** 1995. The plasma membrane of Saccharomyces cerevisiae: structure, function, and biogenesis. Microbiol. Rev. **59:**304–322.
72. **Vezina, C., A. Kudelski, and S. N. Sehgal.** 1975. Rapamycin (AY-22,989), a new antifungal antibiotic. I. Taxonomy of the producing streptomycete and isolation of the active principle. J. Antibiot. (Tokyo) **28:**721–726.
73. **Vilella-Bach, M., P. Nuzzi, Y. Fang, and J. Chen.** 1999. The FKBP12-rapamycin-binding domain is required for FKBP12-rapamycin-associated protein kinase activity and G1 progression. J. Biol. Chem. **274:**4266–4272.
74. **Vojtek, A. B., S. M. Hollenberg, and J. A. Cooper.** 1993. Mammalian Ras interacts directly with the serine/threonine kinase Raf. Cell **74:**205–214.
75. **Weisman, R., and M. Choder.** 2001. The fission yeast TOR homolog, tor1+, is required for the response to starvation and other stresses via a conserved serine. J. Biol. Chem. **276:**7027–7032.
76. **Weissman, Z., R. Shemer, E. Conibear, and D. Kornitzer.** 2008. An endocytic mechanism for haemoglobin-iron acquisition in Candida albicans. Mol. Microbiol. **69:**201–217.
77. **Whiteway, M., and U. Oberholzer.** 2004. Candida morphogenesis and host-pathogen interactions. Curr. Opin. Microbiol. **7:**350–357.
78. **Wilson, R. B., D. Davis, and A. P. Mitchell.** 1999. Rapid hypothesis testing with Candida albicans through gene disruption with short homology regions. J. Bacteriol. **181:**1868–1874.
79. **Wullschleger, S., R. Loewith, and M. N. Hall.** 2006. TOR signaling in growth and metabolism. Cell **124:**471–484.
80. **Zheng, X. F., and S. L. Schreiber.** 1997. Target of rapamycin proteins and their kinase activities are required for meiosis. Proc. Natl. Acad. Sci. U. S. A. **94:**3070–3075.
81. **Zurita-Martinez, S. A., and M. E. Cardenas.** 2005. Tor and cyclic AMP-protein kinase A: two parallel pathways regulating expression of genes required for cell growth. Eukaryot. Cell **4:**63–71.