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RBR1, a Novel pH-Regulated Cell Wall Gene of *Candida albicans*, Is Repressed by *RIM101* and Activated by *NRG1*

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The transcription factor Rim101p of *Candida albicans* has been shown to play a major role in pH-dependent gene regulation. Rim101p is involved in cell wall biosynthesis, since it regulates *PHR1* and *PHR2*, two almost functionally redundant cell wall glycosidases important for adaptation to either neutral or acidic habitats within the human host. To identify additional cell wall components regulated by Rim101p, we performed transcriptional profiling with a cell wall-specific DNA microarray. We showed that Rim101p contributes to the activation of known hypha-specific genes such as *HWP1* and *RBT1* but is also required for repression of the previously uncharacterized potential cell wall genes *RBR1*, *RBR2*, and *RBR3*. Further characterization of *RBR1* revealed that it encodes a small glycosylphosphatidyl inositol protein that is expressed under acidic conditions predominantly at low temperature. Deletion of the gene resulted in a filamentation defect at low pH. Most interestingly, *NRG1*, a transcriptional repressor of hyphal growth in *C. albicans*, was required for *RBR1* expression. The apparently activating effect of *NRG1* observed in this study has not been described before. In addition, we showed that expression of *NRG1* is not only temperature but also pH dependent.

Candida albicans is the most frequent causative agent of candidiasis, which is among the most important nosocomial infections of humans. A key feature of *C. albicans* is its ability to grow in diverse microenvironments of the human body. Examples are the skin and oral and gut mucosae as well as the vaginal mucosa. Each of these niches imposes diverse stresses on the fungus, including nutrient limitation, temperature shifts, and change of pH. A complex network of signaling pathways mediates adaptation of *C. albicans* to these diverse environmental conditions (24). These signaling pathways converge on transcriptional activators such as Efg1p (39, 40) and Nrg1p, which is postulated to reconstitute a DNA-binding repressor complex (30).

The transcription factor Rim101p is crucial for the regulation of genes in response to external pH (33). Little is known about the processes involved in the pH-specific alteration of cell wall composition in *C. albicans*. The transcription of the two cell wall genes *PHR1* and *PRA1* has been shown to be activated in response to neutral pH in a Rim101p-dependent manner (8, 33, 36, 37), whereas Phr2p, encoding a Rim101p-dependent glycosidase with functions equivalent to Phr1p, is part of the cell wall in an acidic environment (10, 29).

RIM101 is the orthologue of *Aspergillus nidulans* PacC, the best studied of the above-mentioned pH-dependent transcription factors (16), and *Saccharomyces cerevisiae RIM101* (23, 41). It contains a conserved DNA-binding Zn^{2+} finger region and is expressed as a preprotein. Rim101p activation at neutral pH depends on members of a proteolytic cascade, *RIM8* and *RIM20* (8). C-terminally truncated forms of the transcription factor lead to pH-independent constitutive activation or repression of Rim101p target genes (8, 13). For *PHR1*, it has recently been shown that direct binding of processed Rim101p to two consensus sites in the promoter results in transcription of the gene (32). On the other hand, *PHR2* is repressed by *RIM101* at neutral pH and becomes constitutively activated in a *rim101*Δ mutant (8, 33), although the promoter of *PHR2* also contains two Rim101p consensus sites.

Similarly, PacC directly activates *ipnA*, an alkaline-expressed gene of the pH-dependent penicillin synthesis pathway and represses the acid-expressed *gabA*, which is subject to carbon catabolite and nitrogen metabolite repression and pH response in *Aspergillus nidulans* (14, 15, 42). *S. cerevisiae RIM101* also has activating and repressing functions, since it plays a major activating role in meiosis and sporulation (23), but a variety of genes have also been shown to be repressed by transcriptional profiling. *S. cerevisiae RIM101* indirectly regulates ion tolerance by repression of *NRG1*, a known repressor of transcription in *S. cerevisiae* and *C. albicans* (20, 21). In *C. albicans*, Nrg1p is known to interact with the transcription factors Tup1p and Mig1p to repress different subsets of hypha-expressed genes (30).

To investigate the *RIM101*-mediated pH-dependent changes in the cell wall of *C. albicans*, we performed transcriptional profiling experiments. We used a cell wall-specific DNA microarray comprising 117 open reading frames related to cell wall synthesis (39). Our results indicate both activating and repressing functions of *RIM101*. One of the genes repressed by *RIM101*, *RBR1*, was characterized in more detail, revealing a novel pH- and temperature-regulated gene from the cell wall of *C. albicans* required for proper hyphal development. Furthermore, our results indicate that *NRG1* plays a mayor role in activating *RBR1* and that *NRG1* expression is regulated in a pH-dependent manner mediated by *RIM101*. These results correlate with induction of the hypha-specific gene *HWP1*. Moreover, *RIM101* contributes to activation of *RBT1*, a hyphal gene repressed by Tup1p independent of Nrg1p (5). These results link pH regulation in *C. albicans* to both Nrg1p-dependent and Nrg1p-independent morphogenetic pathways.

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TABLE 1. *C. albicans* strains used in this study

Strain	Former name	Genotype[a]	Reference
Wild type	SC5314	Clinical isolate	19
CAI4	CAI4	*ura3*::*imm434*/*ura3*::*imm434*	17
*rim101*Δ	CAF3-X	*ura3*::*imm434*/*ura3*::*imm434 rim101*::*hisG*/*rim101*::*hisG*::*URA3*	13
RIM101–1426	CAF3-16-2	*ura3*::*imm434*/*ura3*::*imm434 RIM101*/*RIM101*-(*pBSK*$^+$-*RIM101-1426-URA3*)$_{n>2}$	13
*efg1*Δ	HLC52	*ura3*::*imm434*/*ura3*::*imm434 efg1*::*hisG*/*efg1*::*hisG*::*URA3*	25
*nrg1*Δ	MMC3	*ura3*::*imm434*/*ura3*::*imm434 nrg1*::*hisG*/*nrg1*::*hisG*::*URA3*	31
nrg1-pMET3-NRG1	MMC5	*ura3*::*imm434*/*ura3*::*imm434 nrg1*::*hisG*/*nrg1*::*hisG RP10*::*pMET3-NRG1-URA3*	31
*tup1*Δ	BCA2-9	*ura3*::*imm434*/*ura3*::*imm434 tup1*::*hisG*/*tup1*::*hisG*::*URA3*	3
*rbr1*Δ	HL12-2 and HL14-1	*ura3*::*imm434*/*ura3*::*imm434 rbr1*::*FRT*/*rbr1*::*FRT RP10*::*URA3*	This study[b]
*rbr1*Δ-*RBR1*	HL13-6	*ura3*::*imm434*/*ura3*::*imm434 rbr1*::*FRT*/*rbr1*::*FRT RP10*::*RBR1-URA3*	This study

[a] FRT denotes the FLP recognition sites remaining after excision of the *URA3*-FRT flipper cassette (28).
[b] Mutant strains described in this study were constructed three times independently.

MATERIALS AND METHODS

***C. albicans* strains.** The *C. albicans* strains used in this study are listed in Table 1. DNA microarray experiments were carried out with three different strains. The wild-type strain (SC5314), the homozygous *rim101*Δ deletion strain (CAF3-X *ura3*::*imm434*/*ura3*::*imm434 rim101*::*hisG*/*rim101*::*hisG*::*URA3*) (13) and the *RIM101-1426* strain (CAF3-16-2 *ura3*::*imm434*/*ura3*::*imm434 RIM101*/*RIM101*-[pBSK$^+$-*RIM101-1426-URA3*]$_{n>2}$), which overexpresses dominant active Rim101-1426p (13).

RBR1 deletion mutants were constructed by sequential homologous recombination of CAI4 with a *URA3* flipper cassette (28). One inner and one outer pair of sequences flanking the *RBR1* coding sequence were amplified by PCR. The outer pair of flanking regions (FR1 and FR2) were used for deletion of the first allele; the inner flanking regions (FR3 and FR4) were used for deletion of the second *RBR1* allele. The following primers were used to amplify the outer and inner flanking regions by divergent PCR in a Peltier Thermal Cycler 200 (MJ Research) within 30 cycles at 55°C: FR1_for, 5′-AA*GGGCCC*CACAAAATA AAAGCAGCAGGAA, and FR1_rev, 5′-CCG*CTCGAG*TTCCAACTTTAATC CCGCAC (product length 457 bp); FR2_for, 5′-ATAAGAAT*GCGGCCGC*TT GCCACCAGTCAAATTCAA, and FR2_rev, 5′-*CGAGCTC*CCGAAATGCC ACCATAGTTT (product length 527 bp); FR3_for, 5′-AA*GGGCCC*GTG CGGGATTAAAGTTGGAA, and FR3_rev, 5′-CCG*CTCGAG*TTGTTGTTGT AAGCGAAGCC (product length 563 bp); FR4_for, 5′-ATAAGAAT*GCGGC CGC*TGAATGAGAATGAGGGGGAC, and FR4_rev, 5′-*CGAGCTC*TTGAA TTTGACTGGTGGCAA (product length 565 bp). Embedded in the primer sequences were unique cleavage sites (italics) for directed ligation of the flanking regions into plasmid pSFU1 (28).

FR1 and FR3 were ligated into the vector after ApaI and XhoI digestion, FR2 and FR4 were integrated via NotI and SacI, resulting in plasmids pSFUR1-2 for the outer and pSFUR1-4 for the inner deletion constructs. For reversion of homozygous *rbr1*Δ strains, the coding sequence and promoter region of *RBR1* were cloned into the integrative *C. albicans* expression vector pCaExp (6) with primers 2736f-1_FR1_for (AAGGGCCCCACAAAATAAAAGCAGCAGGAA) and RBR1_RVT_rev (CCGCTCGAGCCGAAATGCCACCATAGTTT). This vector carries the *URA3* gene under its native promoter and was designed for integration into the *RP10* locus. We transformed three independent *rbr1*Δ mutant strains each with pCaExp and pCaExp-*RBR1*. Transformants expressing *URA3* were selected on synthetic complete medium lacking uridine (SC-uri). Single colonies were picked and cultivated for 6 h in SC-uri at 30°C to confirm recombination of the *RBR1* locus by Southern blotting. To monitor mRNA abundance by Northern blot experiments, strains were grown at 30°C in alpha minimal essential medium (α-MEM) buffered to pH 4.5.

Media and growth conditions. All media used in this study contained final concentrations of 100 mM HEPES and 0.1 mM uridine. Desired pHs were adjusted with either 1 M NaOH to pH 7.4 or 1 M HCl to pH 4.5 before filter sterilization. For DNA microarray experiments, cells from overnight cultures grown at 30°C in YPD (Difco) at pH 7.4 and pH 4.5 were pelleted, decanted, and resuspended in the residual medium. Warmed medium of equal pH was inoculated with the cell suspension to an optical density at 600 nm of 0.05. The *rim101*Δ mutant and wild-type reference were grown at pH 4.5; to compare the *RIM101-1426* mutant and wild-type reference strain, both strains were cultivated at pH 7.4. To avoid undesirable effects due to significant changes in pH, it was monitored at the end of each experiment. Fluctuation was not more than 0.2 pH units. When Northern blot experiments were conducted to confirm the DNA microarray data, the strains were cultivated under identical conditions.

Northern blot experiments and phenotypic studies were carried out in YPD, α-MEM (Gibco) and α-MEM without cysteine and methionine containing 2% glucose, and tissue culture medium M-199 (Gibco) with 0.1 mM uridine and 100 mM HEPES. Strains were grown at 25, 29, 30, and 37°C at acidic and neutral pHs as indicated in the Results. Solid medium contained either 2% agar or 0.3% agar for soft agar plates. Heated 4% agar was added to warmed, autoclaved, and filter-sterilized medium up to the final concentration.

Composition of the *C. albicans* cell wall DNA microarray. The cell wall-specific DNA microarray was recently designed and described (39). This array consists of 117 different probes for known genes and not-yet-characterized open reading frames of *C. albicans*; 65 of these genes were designated homologues of cell wall-localized genes in *S. cerevisiae*. Open reading frames in this work are labeled following the assembly 6 names of the Stanford *C. albicans* database (http://www-sequence.stanford.edu/group/candida). In addition, the array comprises genes coding for proteins that are already known to be localized to the cell wall of *C. albicans*. Probes for actin, *ACT1* (X16377), were included as controls.

DNA microarray preparation, isolation of RNA, labeling of cDNA, and hybridization to DNA microarrays. DNA microarray preparation, isolation of RNA, labeling of cDNA, and hybridization to the DNA microarrays were done as previously described (39) with the following modifications: PCR was performed in PCR buffer (100 mM Tris-HCl [pH 8.8], 600 mM KCl, 15 mM $MgCl_2$), containing 1 M betaine (Sigma) and 0.3 μM cresol red (Sigma). After analysis of the PCR products by agarose gel electrophoresis, the amount of liquid was reduced by evaporation overnight at 8°C to achieve a final betaine concentration of at least 2 M before spotting of the probes on polylysine-coated glass slides (12).

Cells for isolation of RNA were cultivated for 6 h, optical density was checked, and the cultures were harvested by brief centrifugation at 1,700 × *g*. Cell pellets were immediately frozen in liquid nitrogen, and the pH of the supernatant was checked. From the frozen cell pellets, total RNA was isolated as described before (39).

Fluorescently labeled cDNA for microarray hybridization was obtained by reverse transcription of 30 μg of RNA with Superscript II (Invitrogen) with either indocarbocyanine-dUTP or indodicarbocyanine-dUTP (Amersham) and hybridized to the DNA microarrays as described before (39).

Analysis of DNA microarray data. DNA microarrays were scanned by epifluorescence microscopy with a GMS 418 array scanner (Genetic Microsystems) and the ImaGene software version 3.0 (BioDiscovery, Los Angeles, Calif.) (39). Data presented are means of duplicates from at least three different experiments. DNA microarray data were largely confirmed by Northern blot analysis under identical conditions (see above, Strains and growth conditions).

Northern hybridization. For Northern hybridization, 15 μg of total RNA was separated by denaturing gel electrophoresis in 1% agarose–morpholinepropanesulfonic acid (MOPS) buffer (0.02 M MOPS, 8 mM sodium acetate, 1 mM EDTA) and 2.2 M formaldehyde. RNA was blotted onto Hybond-N (Amersham) nylon membranes with 20× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate) following standard protocols (35) and UV cross-linked on wet blots twice at 120 mJ in a Stratalinker (Stratagene). Blots were either prehybridized for 1 h at 65°C or frozen at −80°C before further processing. PCR-amplified probes (for primers, see Table 2) were purified and subsequently labeled with [α-^{32}P]dCTP by Klenow reaction with random primers (Stratagene). Hybridization with single probes was performed for at least 6 h, followed by three washing steps in 1× SSC–0.1% sodium dodecyl sulfate (SDS). Blots were exposed to a phosphor screen (Molecular Dynamics) for 24 to 72 h. Screens were scanned with a PhosphorImager (Molecular Dynamics), and individual bands were quantified with ImageQuant 5.2 (Molecular Dynamics). Signals were either quantified

TABLE 2. Synthetic oligonucleotide primers used for Northern hybridization

Gene	Primer	Sequence
ACT1	act1for	TTTCCAACTGGGACGATA
	act1rev	TCTTGGACAAATGGTTGG
ALS1	als1for	ACTGGTTGCTACTACACA
	als1rev	GGACAATAATGTGATCAA
ALS5	2848f + 3bfor	ACTGCCGATGGTGTTAAA
	2848f + 3brev	TGGTGCCACAAAAAGAGT
HWP1	2426for	CTAAACCAGCTGCTCCAAAAT
	2436rev	GTTGTTACCAGCACCTTCAAA
NRG1	NRG1for	ATGCTTTATCAACAATCATATCC
	NRG1rev	CTATACTAGGCTCTTGGTG
PHR1	phr1for	TTGCAATGTCCAGGAACT
	phr1rev	CTGCCACTAGCACTAGCC
PHR2	phr2for	GAATCTGCCTCCTCCATT
	phr2rev	AATGAAGCAGAACCACCA
RBR1	forRBR1prox	CTACCAGCACCAGCGACAG
	revRBR1 down	CTGTTGGTGTGGGTTGTGAG
RBR2	RBR2proxfor	AACAGGGGCAACAAGTTCAC
	RBR2downrev	AGTTTGGATTCGACTGTGGG
RBR3	1477for	CCCTGCCTTCCTTAACATCGT
	1477rev	TAAAAATTTTGCGATGTGGAA
RBT1	2768f + 2for	ACTGCCGATGGTGTTAAAT
	2768f + 2rev	TGGTGCCACAAAAAGAGTT
RIM101	RIM101/1–23	ATGGGTAACAGTCCCCATTCCTC
	RIM101/1048–1027	GGCTTCAATGGGACATGGACTC
TUP1	CaTUP1for	ATGTATCCCCAACGCACC
	CaTUP1rev	TTATTTTTTGGTCCATTTCC

after normalization to actin mRNA levels or related to ethidium bromide-stained 18s and 28s rRNA bands, as indicated in the Results.

Isolation of chromosomal DNA and Southern hybridization. Chromosomal DNA of *C. albicans* strains was isolated as described previously (27), digested with EcoRI and PstI, and separated on 0.8% agarose. Blotting onto nylon membranes in 20× SSC and hybridization with an [α-^{32}P]dCTP randomly labeled gene-specific probe was done by standard protocols (35). Membranes were exposed on a phosphor screen (Molecular Dynamics) for 12 h. They were scanned and visualized as described for Northern hybridization.

RESULTS

DNA microarray analysis to identify novel *RIM101*-regulated cell wall genes in *C. albicans*. The cell wall of *C. albicans* protects the fungus from its environment and is crucial for host-pathogen interactions. Thus, the cell wall has to be adapted and remodeled constantly to cope with various environmental conditions. The pH-dependent transcription factor Rim101p is known to regulate *PHR1* and *PHR2*, two glycosidases involved in cell wall biosynthesis in *C. albicans* (29). To identify additional *RIM101*-regulated cell wall genes, we performed transcriptional profiling with a cell wall-specific DNA microarray. The array has been described previously (39) and consists of known *C. albicans* cell wall genes and 65 open reading frames (ORFs) with significant homology to already-characterized cell wall proteins in *S. cerevisiae*. Microarray experiments were carried out with a *rim101*Δ mutant and strain *RIM101-1426*, overexpressing C-terminally truncated, dominant active Rim101-1426p (Table 1). These mutant strains were compared to wild-type cells grown in 30°C YPD at pH 7.4 and pH 4.5, respectively. In order to focus on gene regulation induced by pH changes, all strains were grown under conditions favoring blastospore morphology (see Materials and Methods). Thus, none of the strains showed filamentous growth when the cells were harvested for RNA isolation after 6 h of cultivation (data not shown).

***RIM101* activates and represses cell wall genes of *C. albicans*.** Our results indicate that *RIM101* is required for both activation and repression of transcription. In this focused approach, looking at cell wall genes only, we predominantly found *RIM101*-repressed genes. In total, comparing the *rim101*Δ mutant and the *RIM101-1426* mutant with the wild type, 32 genes out of 117 were differentially regulated by *RIM101*. All of these genes showed at least a 2.5-fold change in expression rate in three independent experiments. *RIM101* was involved in repression of 23 potential cell wall genes, whereas nine genes turned out to be *RIM101* activated under the conditions tested. Table 3 gives an overview of genes regulated by *RIM101*, as revealed by transcriptional profiling.

Genes activated by Rim101p. Transcriptional profiling revealed cell wall genes activated by *RIM101*. Consistent with existing data, *PHR1* and *SKN1*, another putative cell wall glycosidase gene, were strongly upregulated in the *RIM101-1426* strain compared to the wild-type strain (32) (Table 3). Notably, *HWP1* and *RBT1* were revealed to be induced in a *RIM101-*

TABLE 3. *C. albicans* cell wall genes activated and repressed by *RIM101*

Category	Gene (accession no.)/ORF	Fold reduction		Mean deviation[a]
		RIM101-1426/ wild type (pH 4.5)	*rim101*Δ/ wild type (pH 7.4)	
Activated genes	*PHR1* (AF247190)	32.1		19.6
	HWP1 (U64206)/*ECE2* (AF001978)	25.8		20.0
	ALS1 (L25902)	12.8		8.8
	orf6.7834	8.0		5.8
	SKN1 (D88491)	5.6		1.1
	ALS5 (AF068866)	4.2		2.7
	orf6.1982	3.4		1.1
	RBT1 (AF254142)	3.1		0.7
	orf6.1230/orf6.1231/ orf6.1232	2.9		0.8
Repressed genes	*PHR2* (AF011386)		15.2	2.9
	RBR1 (orf6.6747)		15.2	10.9
	PHO genes (*orf*6.4212/ orf6.1652/orf6.1832)		9.2	2.3
	PHO11 (orf6.1832)		6.7	1.4
	RBR2 (orf6.6744)		5.2	1.3
	RBR3 (orf6.1159)		4.8	2.1
	CHS1 (X52420)		4.5	1.9
	orf6.4590		4.5	2.0
	INT1 (U35070)		4.1	1.6
	orf6.3954		3.6	0.9
	orf6.4388		3.6	0.6
	orf6.3476		3.4	0.7
	orf6.2804		3.4	0.7
	orf6.4736/orf6.4737		3.1	1.0
	orf6.799		3.0	0.7
	orf6.7718		3.0	1.6
	orf6.8118		2.9	0.5
	orf6.474/orf6.475		2.7	0.5
	orf6.6972		2.7	0.5
	ALS4 (AF024586, AF024584)		2.7	0.8
	KRE6 (D88490)		2.6	0.4
	orf6.5422/orf6.5423/ orf6.537/orf6.538		2.6	0.7
	orf6.7977		2.6	0.6
	orf6.4073/orf6.4765		2.5	0.7

[a] The cited mean deviations were derived from least three experimental repeats in duplicate.

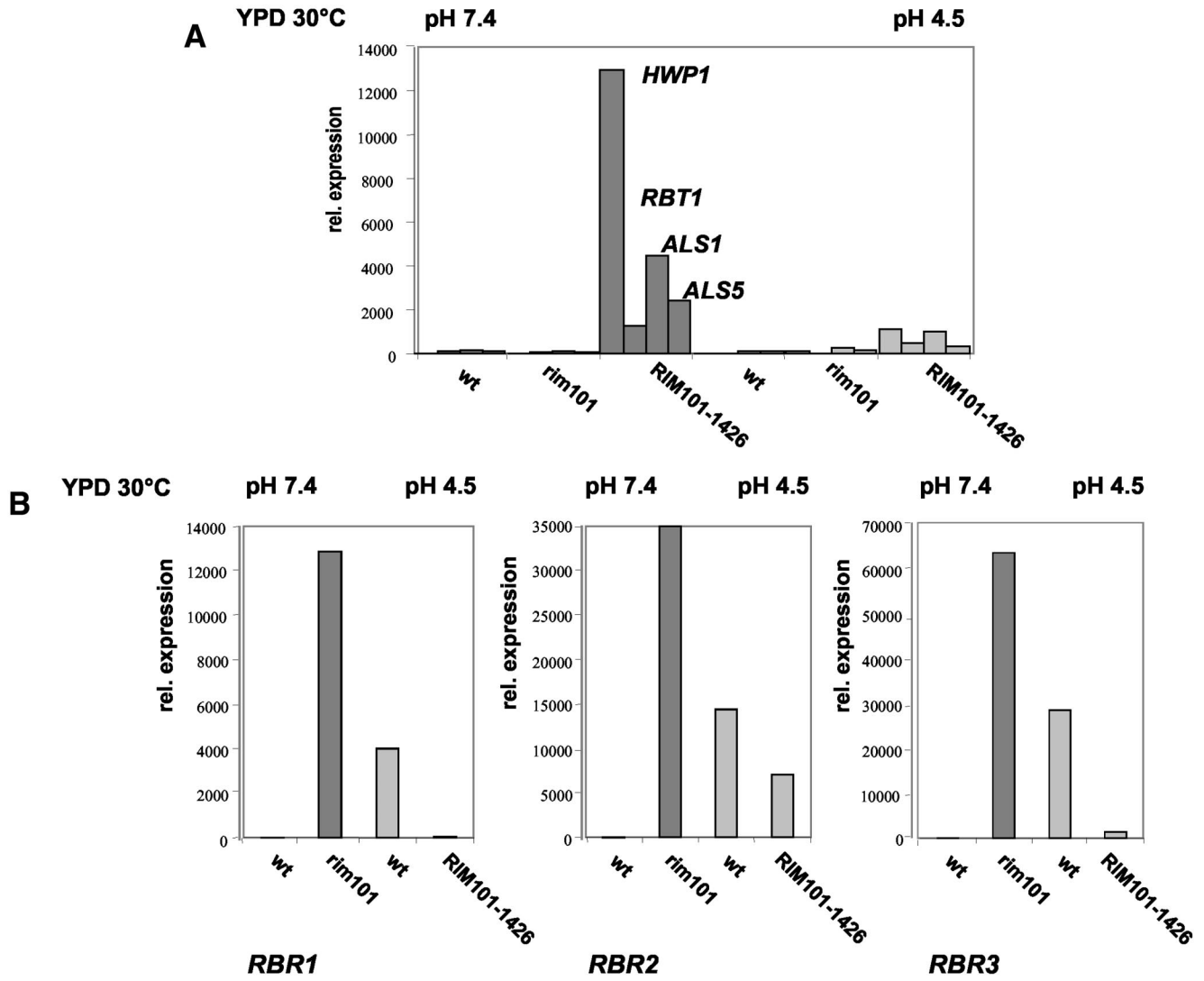

FIG. 1. Rim101p activates and represses cell wall genes of *C. albicans*. (a) Activation of hyphal genes by *RIM101*. (b) Repression of *RBR1*, *RBR2*, and *RBR3* by *RIM101*. RNA for Northern blotting was prepared from the wild-type strain SC5314 and the *RIM101-1426* and *rim101*Δ mutants grown for 6 h in YPD with 100 mM HEPES at 30°C. Media were adjusted to pH 7.4 for SC5314 and the *rim101*Δ mutant (dark bars) or to pH 4.5 for SC5314 and the *RIM101-1426* mutant (light bars). Quantification of mRNA abundance was done by normalizing hybridization signals to the *ACT1* signal of SC5314 at pH 7.4.

dependent way in blastospores (YPD, 30°C, pH 7.5). Overexpression of Rim101-1426p under these conditions strongly induced transcription of *HWP1*, *RBT1*, *ALS1*, and *ALS5* at pH 7.4. At pH 4.5, this effect was much weaker, indicating that additional pH-dependent factors may be relevant for activation of these cell wall genes by Rim101p (Fig. 1a). This finding was unexpected, because Hwp1p is a well-characterized hyphal wall protein (38). Rbt1p was defined as a cell wall protein repressed by Tup1p, a transcription factor negatively regulating hyphal growth (2), and expression of both genes has been shown to depend on Efg1p (4, 24).

Genes repressed by Rim101p. As expected, *PHR2* was strongly repressed in the presence of active Rim101p under all conditions tested (8, 33) (Table 3). Additionally, *PHO11* was repressed by *RIM101*, as recently revealed by transcription analysis of *C. albicans* by others (32). This result also parallels the situation in *S. cerevisiae* (21) and in *A. nidulans* (44). In addition, several as yet uncharacterized ORFs were strongly repressed. Three of the ORFs repressed by *RIM101* were examined further and named *RBR1* (orf6.6747), *RBR2* (orf6.6744), and *RBR3* (orf6.1159). To confirm our microarray data and to quantify transcription of these genes, we subsequently performed Northern blot analysis (Fig. 1b). *RBR1*, *RBR2*, and *RBR3* were expressed under acid conditions, and no significant expression was detected in the wild type at neutral pH. Furthermore, the *RBR* genes were repressed by dominant active *RIM101-1426* at acidic pH and strongly upregulated in *rim101*Δ mutant strains under all conditions (Fig. 1b and Fig. 4).

Following in silico examination, all three *RIM101*-repressed genes carried an approximately 20-amino-acid N-terminal signal sequence for translocation into the endoplasmic reticulum and a C-terminal hydrophobic transmembrane domain (http://www.cbs.dtu.dk/services/SignalP). All of the novel *RBR* protein sequences also enclose a hydrophobic N-terminal domain

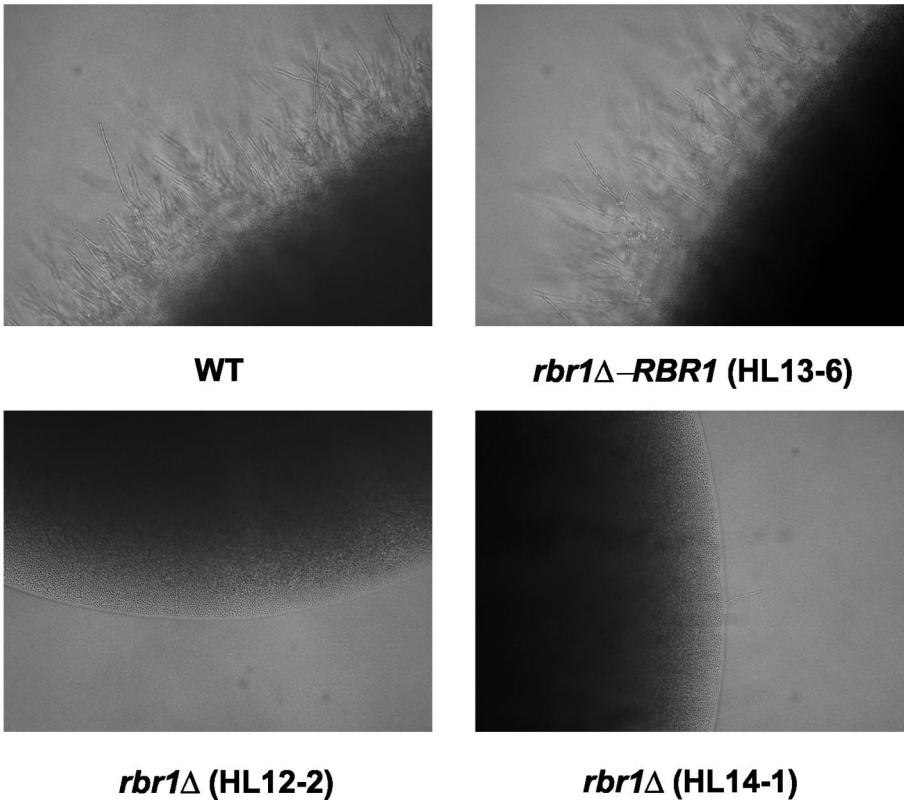

FIG. 2. pH-dependent filamentation defect of *rbr1*Δ mutants on M-199 soft agar. The independent *rbr1*Δ mutants HL12-2 and HL14-1 are shown in the lower panels; HL13-6 is derived from HL12-2. Strains were precultured in SC-uri for 24 h and pelleted by brief centrifugation. Then 5 μl of concentrated cell suspension was spotted onto the agar surface. Plates were incubated for 72 h at 30°C.

(http://129.194.185.165/dgpi/DGPI_demo_en.html). The hydrophobic tail of *RBR3* was predicted to be too short for location in the plasma membrane, most likely because orf6.1159 was annotated as a C-terminally incomplete ORF in the Stanford *C. albicans* database. The predicted domains are characteristic of glycosylphosphatidyl inositide (GPI)-anchored cell wall proteins, and they are followed by an upstream omega site sequence defining the cleavage site for attachment of the GPI anchor and further determinants for location in either the cell wall or the plasma membrane (7, 11, 18). Rbr1p (111 amino acids) and Rbr2p (168 amino acids) have potential cleavage sites at positions 81 and 143, respectively; Rbr3p (560 amino acids) shows a potential cleavage site at position 540. In addition, the protein sequence, excluding C- and N-terminal hydrophobic sequences, exhibits several sites with a high O-β-glycosylation potential typical of fungal cell wall proteins (http://www.cbs.dtu.dk/services/YinOYang).

Searching the pro- and eukaryotic genome databases at the National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov/BLAST), we found no significant homology of the *RBR* genes with genes in *S. cerevisiae* or any other organisms. A protein query for nearly exact matches (BLASTP 2.2.6) at NCBI revealed 40% identity of Rbr3p with Hyr1p of *C. albicans*, a GPI-anchored nonessential cell wall protein abundant in hyphae (1).

***rbr1*Δ mutant strain shows a pH-dependent filamentation defect.** Since *RBR1* showed the strongest expression among the novel *RIM101*-repressed genes, we characterized this gene further. The open reading frame (orf6.6747) encoding *RBR1* is 336 bp long and predicted to encode a GPI-anchored protein of only 111 amino acids in length. For functional characterization of the potential cell wall protein Rbr1p in *C. albicans*, we constructed three independent *rbr1*Δ mutant strains by transformation and homologous recombination of CAI4 with the *URA3* flipper cassette (28). Strains were routinely first selected for transformants on SC-uri and in a second step after induction of the FLP recombinase for *ura3* mutant colonies. Homozygous *rbr1*Δ mutants were reverted either with the *URA3* gene only or with *URA3* and *RBR1* under its native promoter (Table 1).

Southern and Northern blot analyses confirmed the genotype and the absence of *RBR1* transcription in the homozygous *rbr1*Δ mutant strains. Heterozygous and homozygous *rbr1*Δ mutants showed no growth defects under any conditions used. Since *RBR1* was expressed prominently at low pH (Fig. 1b), we expected potential alterations in growth, resistance to cell wall stress, or filamentation defects especially under these conditions. We examined growth and filament induction of the *rbr1*Δ mutants in the presence of hydrogen peroxide, Calcofluor White, and 0.3 M NaCl on different solid media at 25, 30, and 37°C. No distinct phenotype was observed on media with 2% agar, but we were able to detect a filamentation defect of *rbr1*Δ strains on soft agar containing 0.3% agar. On M-199 soft agar buffered to pH 4.5, *rbr1*Δ did not show filamentous growth within 3 days of incubation (Fig. 2), while the wild-type and the *RBR1* revertant strains readily induced filamentation after 24 h

at 30°C. Under the same conditions at pH 7.4, the *rbr1*Δ strain did induce filaments after 24 h (data not shown).

This defect in filament induction implied that *RBR1* was especially needed in the cell wall of *C. albicans* in an acidic environment, consistent with its expression profile (Fig. 1b). After 5 days on acidic M-199 soft agar at 30°C, possibly as an unrelated starvation response, *rbr1*Δ mutant strains also started to induce hyphae, growing out of the colonies from the center to the edge and downwards into the agar (data not shown). When tested in liquid media, no differences in growth or filamentation rate were detected between the wild-type and *rbr1*Δ mutant strains (data not shown), indicating that surface-mediated effects may be responsible for the observed phenotype.

***NRG1* activates expression of *RBR1*.** Since Rbr1p seemed to be involved in the dimorphic switch, we asked if, in addition to Rim101p, other transcriptional regulators of morphogenesis in *C. albicans* were involved in *RBR1* regulation. Therefore, we examined expression of *RBR1* in the *efg1*Δ, *nrg1*Δ, and *tup1*Δ mutant strains at pH 4.5 at 25°C and pH 7.4 at 37°C in hypha-inducing medium (Fig. 3a). Notably, we found expression of *RBR1* to be strongly reduced in the *nrg1*Δ mutant at pH 4.5, while wild-type transcript levels were detected in the *efg1*Δ and *tup1*Δ mutants. At neutral pH, we did not detect significant *RBR1* expression in either the wild type or the mutant strains tested.

The *nrg1*Δ mutant strain MMC4 was reverted with *NRG1* under the control of the *MET3* promoter. In this revertant strain, transcription of *NRG1* and *RBR1* was restored in α-MEM without cysteine and methionine (Fig. 3b). These data implied that Nrg1p directly or indirectly activated *RBR1* transcription, while Efg1p and Tup1p were not involved in *RBR1* regulation under the conditions tested. In parallel, we looked at the influence of Efg1p, Nrg1p, and Tup1p on *PHR2* expression (Fig. 3a). *PHR2*, like *RBR1*, is acid expressed (29), but we did not find Nrg1p to be required for activation of *PHR2*. These results demonstrated that although both *RBR1* and *PHR2* are acid-induced genes, they are activated by different mechanisms. Furthermore, these data showed that *RIM101* transcription did not depend on the presence of the transcription factor Efg1p, Nrg1p, or Tup1p.

Expression of *NRG1* is repressed by *RIM101* and negatively related to temperature. Northern blot analysis revealed that the cell wall gene *RBR1* was inversely regulated by the two transcription factors Rim101p and Nrg1p (Fig. 1b and 3). Furthermore, expression of *RIM101* was independent of *NRG1* (Fig. 3). We additionally performed experiments to examine whether expression of *NRG1* depended on *RIM101*. Figure 4a demonstrates that both *NRG1* and *RBR1* were expressed in a pH-dependent manner, directly correlated to the activity of *RIM101*. In the wild type, *NRG1* and *RBR1* were strongly expressed at pH 4.5, while very low transcript levels were detected at pH 7.4. The presence of dominant active Rim101p significantly reduced expression of *NRG1* and *RBR1* at acidic pH, whereas deletion of *RIM101* led to activation of *NRG1* and *RBR1* at neutral pH under all conditions tested.

Searching the promoter regions of *NRG1* and *RBR1* for Rim101p binding sites (CCAAGAAAA) (32), we found consensus sequences for Rim101p binding at positions −1302 bp for *NRG1* and −1400 bp for *RBR1*. To prove that Rim101p binds directly to these consensus sequences, further studies will be necessary.

In addition, the Northern blot experiments revealed that expression of *NRG1* and *RBR1* was negatively correlated to temperature (Fig. 4a). Compared to the wild type at pH 4.5, 25°C, expression of *NRG1* and *RBR1* was strongly reduced at 37°C and pH 4.5. This result indicated a temperature-dependent factor restricting *NRG1* expression in addition to the Rim101p-dependent and therefore pH-modulated repression of *RBR1* and *NRG1*. Since Nrg1p is known to repress a variety of hypha-specific genes, we moreover did explore Rim101p-mediated induction of *HWP1* under hypha-inducing conditions. *HWP1* transcription was induced at 25 and 37°C, when Rim101p-mediated repression of Nrg1p was observed (Fig. 4b). The same effect was also found for both *HWP1* and *RBT1* in M-199 at 29 and 37°C (Fig. 5a). This Northern blot analysis additionally showed that temperature had a major effect on the expression of *HWP1* and *RBT1*, which was also detected for the expression of *NRG1*. The corresponding phenotype of the wild-type and *RIM101* mutant strains in M-199 (37°C) is shown in Fig. 5b. The *RIM101-1426* mutant exhibited filamentous growth at acidic pH, as expected, but we also microscopically observed flocculation of *C. albicans* in response to *RIM101-1426* overexpression at neutral pH.

In conclusion, our data demonstrated that active *RIM101* was able to repress *NRG1*, as has been shown for *NRG1* in *S. cerevisiae* (20). We also found that Nrg1p, a repressor of hyphal genes, showed an activating function towards the acid-expressed cell wall gene *RBR1* in *C. albicans*. Moreover, our data led to the hypothesis that repression of *NRG1* by *RIM101* is responsible for pH-dependent induction of the cell wall protein Hwp1p and that Rim101p in parallel activates Rbt1p.

DISCUSSION

Repression and activation of cell wall genes by *RIM101*. The pH-dependent transcription factor Rim101p is known to act as both a transcriptional repressor and activator. In accordance with this, our transcriptional profiling experiments to detect novel downstream targets of Rim101p in *C. albicans* revealed a set of cell wall genes that are either repressed or induced by Rim101p in response to changes in the environmental pH (Table 1).

***RIM101* is responsible for pH-dependent activation of hyphal genes.** In our experimental approach, we focused on pH-dependent cell wall gene regulation by Rim101p and attempted to avoid activation of the developmental program inducing hyphae by growing all strains as blastospores (see Materials and Methods). Nevertheless, our microarray data reveal significant activation of the hypha-specific genes *HWP1*, *RBT1*, *ALS1*, and *ALS5* by *RIM101* in a pH-dependent way (Table 3 and Fig. 1a). *RIM101* has been shown to contribute to filamentation of *C. albicans* on a phenotypic level and on a molecular level to the regulation of *HWP1* (8, 9, 13). Our studies show that Rim101p also contributes to the regulation of additional hypha-specific cell wall genes (Fig. 1a and 5a). These results apparently correlate with the *RIM101-1426* phenotype, which is characterized by filamentous growth at acidic pH and an excess adhesion phenotype at neutral pH and 37°C (Fig. 5b).

At acidic pH (pH 4.0), activation of both *RBT1* and *HWP1* by *RIM101-1426* was significant but not as strong as at pH

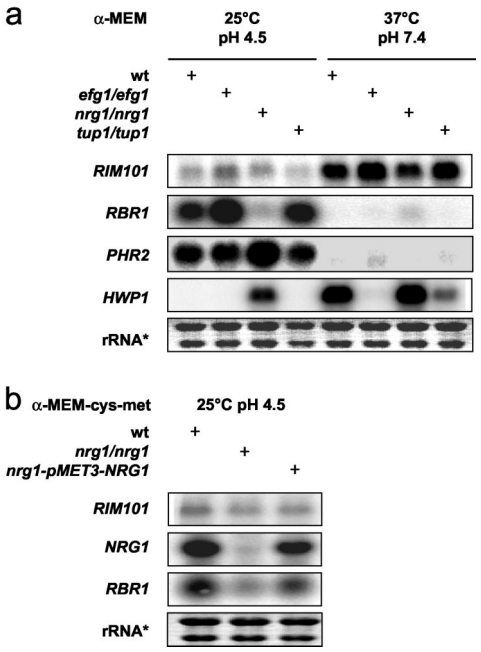

FIG. 3. (a) *RBR1* expression is activated by *NRG1*. Transcript levels of *RBR1*, *PHR2*, and *HWP1* in the wild type (wt) and *efg1*Δ, *nrg1*Δ, and *tup1*Δ mutant strains were determined by Northern blotting. RNA derived from strains grown in α-MEM at pH 4.5 (lanes 1 to 4) and pH 7.4 (lanes 5 to 8) for 4 h under noninducing and hypha-inducing temperatures of 25 and 37°C, respectively. (b) Reversion of the *nrg1*Δ mutant strain with p*MET3-NRG1* restores *RBR1* expression. The wild type and *nrg1*Δ, and *nrg1*-p*MET3-NRG1* mutants were grown for 4 h in α-MEM at pH 4.5 without cysteine and without methionine. rRNA*, ethidium bromide-stained loading control.

8.0 (Fig. 5a and 1a). In addition, we found that the level of *RIM101*-mediated activation depended on the temperature. Higher temperature in general results in stronger activation of *HWP1* and *RBT1*. Obviously, additional factors besides *RIM101* contribute to the pH regulation as well as the tempera-

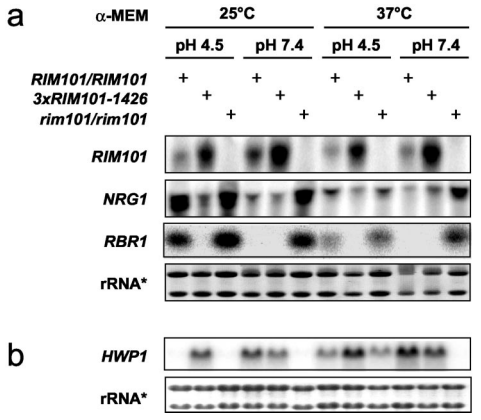

FIG. 4. (a) Expression of *NRG1* and *RBR1* is *RIM101* and temperature dependent. (b) pH-dependent repression of *NRG1* correlates to Rim101p-dependent expression levels of *HWP1*. Wild-type SC5314 and *RIM101-1426*, and *rim101*Δ mutants were grown for 6 h in α-MEM with 100 mM HEPES buffered to pH 4.5 and 7.4 at 25 and 37°C to isolate RNA for Northern blot analysis. rRNA*, ethidium bromide-stained loading control.

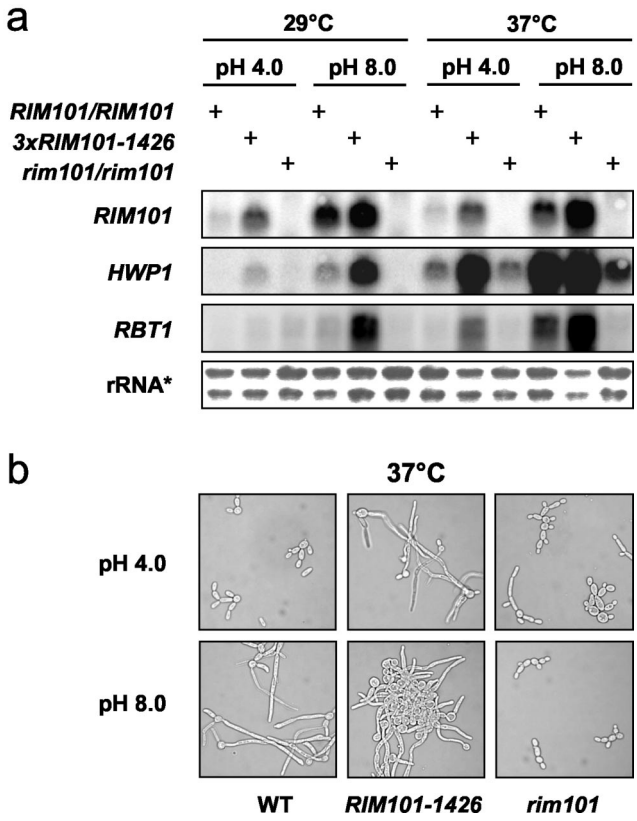

FIG. 5. Expression of *HWP1* and *RBT1* is activated by *RIM101* and correlated to the *RIM101-1426* overexpression phenotype. (a) *RIM101*-dependent expression of *HWP1* and *RBT1* in wild-type SC5314 and the *RIM101-1426* and *rim101*Δ mutants was determined by Northern blotting. RNA was derived from cells grown in M-199 with 100 mM HEPES at pH 4.0 and 8.0 for 4 h. The pH and temperature values are indicated. rRNA*, ethidium bromide-stained loading control. (b) Phenotypes of the strains grown for Northern blot analysis after 4 h in M-199 at 37°C and pH 4.0 (upper panel) or pH 8.0 (lower panel).

ture-mediated induction of hyphal genes. A pH-responsive pathway defined by Mds3p that could participate in inducing the pH-related effects observed has recently been identified (9).

EFG1 is required for expression of Hwp1p, Rbt1p, and Als1p (4, 22). Repression of *HWP1* has been shown to depend on both Nrg1p and Tup1p, while *RBT1* seems to be mainly controlled by Tup1p (2, 4). Our experiments revealed that expression of *RBT1* depended on active Rim101p (Fig. 5a) and was strongly induced at neutral pH and higher temperatures in the wild type. Under these conditions, equal levels of *TUP1* mRNA were detected in the wild-type and *rim101*Δ stains (data not shown), and expression of *RIM101* was independent of Tup1p (Fig. 3a). These results suggest that *RIM101*-dependent activation of *RBT1* is not mediated by repression of *TUP1* but that Rim101p, in addition to Efg1p, functions as an activator of *RBT1* in *C. albicans*.

Our data furthermore showed that Rim101p expression was independent of Efg1p (Fig. 3a). Thus, Rim101p and Efg1p seem to independently converge on the regulation of several hypha-specific genes and precisely modulate gene expression in order to adapt to distinct external conditions. These findings are in agreement with a proposed regulation of the hypha-specific genes such as *HWP1*, *RBT1*, and *HYR1* by multiple

transcription factors with DNA-binding capacity (22). Similar complex regulation mechanisms for the expression of cell wall proteins were also observed for Flo11p, a protein necessary for pseudohyphal differentiation in *S. cerevisiae* (26, 34).

Repression by *RIM101*. The vast majority of the genes identified in our experiments are repressed by Rim101p (Table 1). In *S. cerevisiae*, *RIM101* has also been shown to have significant repressing functions in gene regulation (20). Recently, genomewide transcriptional profiling experiments identified 13 genes that are repressed by *RIM101* (32). Two of these genes (*PHR2* and *PHO11*) were also identified in the course of our experiments focused on cell wall genes. The three most strongly repressed and so far uncharacterized ORFs identified in our screen were named *RBR1*, *RBR2*, and *RBR3* (repressed by Rim101p) and encode a set of GPI-anchored proteins with so far unknown function. All three genes are expressed under acidic conditions and repressed by *RIM101* at neutral pH. Similar to the regulation of *PHR2* (13), deletion of *RIM101* resulted in constitutive expression of the *RBR* genes, and dominant active *RIM101-1426* strongly repressed these genes at every pH tested (Fig. 1b and 4a). Therefore, *RIM101* can be considered a repressor of *RBR1*, *RBR2*, and *RBR3*. An in silico promoter analysis of *RBR1* revealed a consensus sequence for Rim101p binding at position −1400 bp, while no established binding site for the transcription factor was found 2 kb upstream of the other two Rim101p-repressed genes.

Functional characterization of *RBR1*. To functionally characterize *RBR1*, we deleted the gene in *C. albicans* and investigated the phenotype of the resulting strain under various conditions, including cell wall stress and osmotic stress and different hypha-inducing media. Deletion of *RBR1* results in a filamentation defect on M-199 soft agar buffered to pH 4.5 (Fig. 2), indicating a function in hyphal development under acidic conditions. The presence of other acid-expressed genes such as *RBR2* and *RBR3* may have compensated for a more severe phenotype.

The phenotype observed is in accordance with the expression pattern of *RBR1*, which displays strong transcription at acidic pH. Our results show that Nrg1p is required for *RBR1* expression. *NRG1* transcription parallels the temperature- and pH-related regulation of *RBR1* (Fig. 4a). This is surprising because so far Nrg1p has only been described as acting as a transcriptional repressor, most likely by recruiting Tup1p to the promoters of target genes, e.g., *HWP1* (31). The transcription factors Efg1p and Tup1p show no effect on *RBR1* expression (Fig. 3a). Although this is the first time that an apparently activating role for Nrg1p in *C. albicans* has been reported, data resulting from transcriptional profiling experiments indicate this activating function of Nrg1p for other genes as well (30). In contrast to *RBR1*, activation of *PHR2* under acidic conditions did not require *NRG1* under the conditions tested, and thus different regulation mechanisms are present for these two acid-expressed genes. Since GPI proteins, as shown for Hwp1p and Als1p, are involved in tissue adhesion by and virulence of *C. albicans*, these proteins might be targets for new antifungal substances (43).

***RIM101* regulates the transcriptional repressor *NRG1*.** Our studies revealed coregulation of *RBR1* by Rim101p and Nrg1p. Further investigations to identify cross talk between Rim101p-dependent pH signaling and *NRG1* showed that active Rim101p represses the transcription of *NRG1* (Fig. 4a), while deletion of

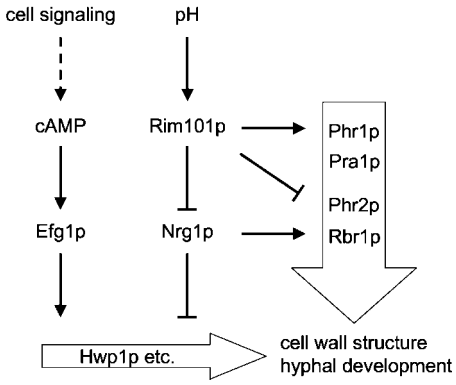

FIG. 6. Model of Rim101p cross talk with the *NRG1* pathway. Integration of external pH signals into other signaling pathways is mediated by *RIM101*-dependent repression of Nrg1p. The presence of active Rim101p at neutral pH has three consequences: first, direct activation of pH-specific cell wall genes (*PHR1*, *PRA1*), second, direct or indirect repression of acid-expressed cell wall genes (*PHR2*, *RBR1*) and third, enhanced expression of hypha-specific genes by repression of *NRG1*. *C. albicans* is thus able to integrate pH signals via Rim101p and Nrg1p with other signaling pathways required, e.g., for morphogenesis to react specifically to changes in environmental conditions.

NRG1 had no significant influence on *RIM101* transcripts (Fig. 3a and b). This indicates that Rim101p acts as a repressor of *NRG1*, as has been reported for *S. cerevisiae* (20). Although our data suggest that Rim101p functions similar to *S. cerevisiae* Rim101p, ectopic expression of Rim101p in an *S. cerevisiae* *rim101*Δ mutant did not complement its invasive growth defects (data not shown). A recent study on Rim101p binding specificity also involved transcriptional profiling of wild-type and *rim101*Δ strains (32). The authors indicate that transcriptional profiling did not reveal activation of *NRG1* or Nrg1p-repressed genes in the *rim101*Δ mutant compared to the wild type (M-199, 28°C, and pH 7.4). The Northern blot experiments shown in Fig. 5a were performed under similar conditions (M-199, 29°C, and pH 8.0) and display a relatively weak induction of the *NRG1*-repressed gene *HWP1* in the wild-type cells. This might explain why significant downregulation of *NRG1*-repressed genes versus wild-type expression could not be detected in DNA microarray experiments under the conditions chosen. We observed that the effect of Rim101p on *NRG1* was most prominent at lower temperatures in α-MEM (Fig. 4a).

Our results suggest that Rim101p not only acts as a transcriptional activator of *PHR1* but also significantly controls gene expression through downregulation of the transcriptional repressor Nrg1p (Fig. 4a). This leads to induction of the hypha-specific gene *HWP1*, as has been shown before (4, 5). Furthermore, the expression of *RBT1* strongly correlates with Rim101p activity (Fig. 5a). High levels of Nrg1p at acidic pH suppress filamentous growth under these conditions (31). Thus, derepression of *NRG1* by deletion of *RIM101* corresponds to the filamentation defect described for the *rim101*Δ mutant at neutral pH (8), while enhanced adhesion and induction of hyphae in the *RIM101-1426* strain (13) reflect downregulation of *NRG1*. How Nrg1p in concert with Rim101p directly or indirectly mediates regulation of *RBR1* expression is still unclear and will be the subject of further research.

Our results document that Rim101p is woven into the com-

plex network of signaling pathways required for morphogenesis (Fig. 6), giving *C. albicans* the ability to integrate pH signals with other environmental signals to react specifically to external stresses.

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