

Transcriptional profiling in *Candida albicans* reveals new adaptive responses to extracellular pH and functions for Rim101p

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Summary

The human pathogen *Candida albicans* grows and colonizes sites that can vary markedly in pH. The pH response in *C. albicans* is governed in part by the Rim101p pathway. In *Saccharomyces cerevisiae*, Rim101p promotes alkaline responses by repressing expression of *NRG1*, itself a transcriptional repressor. Our studies reveal that in *C. albicans*, Rim101p-mediated alkaline adaptation is not through repression of *CaNRG1*. Furthermore, our studies suggest that Rim101p and Nrg1p act in parallel pathways to regulate hyphal morphogenesis, an important contributor to virulence. To determine the wild-type *C. albicans* transcriptional response to acidic and alkaline pH, we utilized microarrays and identified 514 pH-responsive genes. Of these, several genes involved in iron acquisition were upregulated at pH 8, suggesting that alkaline pH induces iron starvation. Microarray analysis of *rim101*^{-/-} cells indicated that Rim101p does not govern transcriptional responses at acidic pH, but does regulate a subset of transcriptional responses at alkaline pH, including the iron acquisition genes. We found that *rim101*^{-/-} cells are sensitive to iron starvation, which suggests that one important aspect of the Rim101p-dependent alkaline pH response is to adapt to iron starvation conditions.

Introduction

Candida albicans is a commensal organism that colonizes the mucosal surfaces of the oral-pharyngeal, gastrointestinal (GI) and urogenital tracts of most humans. In susceptible hosts, *C. albicans* is an opportunistic pathogen of

these mucosal surfaces. However, *C. albicans* can also enter the bloodstream to cause systemic infections that are extremely life threatening with a mortality rate of ≈40% despite the use of available anti-fungal therapies (Edmond *et al.*, 1999).

The sites that *C. albicans* colonizes as a commensal can vary in pH, both temporally and spatially. The pH of the oral cavity varies markedly resulting from changes in diet, the metabolism of other microflora and salivary flow. The GI tract shows dramatic spatial differences in pH, from extremely acidic in the stomach (pH 2), less acidic in the duodenum (pH 5), to alkaline in the intestine (pH 7.7). Systemic candidiasis can arise from endogenous organisms in the GI tract that escape into the bloodstream (pH 7.4) (Krause *et al.*, 1969; Reagan *et al.*, 1990). Thus, as a commensal and a pathogen, *C. albicans* must adapt to, and thrive in, diverse environmental pH.

The ability to adapt to extracellular pH is required for *C. albicans* pathogenesis (Davis, 2003). Two cell wall proteins, Phr1p and Phr2p, are differentially expressed in response to environmental pH. Phr1p is expressed at pH ≥ 5.5; Phr2p is expressed at pH ≤ 5 (Saporito-Irwin *et al.*, 1995; Muhlschlegel and Fonzi, 1997). Furthermore, these proteins are essential for pathogenesis in host sites where they are expected to be expressed: Phr1p is essential for systemic candidiasis (pH 7.4) and Phr2p is essential for vaginal candidiasis (pH 4) (Ghannoum *et al.*, 1995; De Bernardis *et al.*, 1998). The pH-dependent expression of *PHR1* and *PHR2* is governed by a signal transduction pathway that culminates with activation of the zinc finger transcription factor Rim101p (Ramon *et al.*, 1999; Davis *et al.*, 2000a). Rim101p induces *PHR1* and represses *PHR2* expression at alkaline pH and is required for wild-type levels of virulence in a haematogenously disseminated murine model of systemic candidiasis (Davis *et al.*, 2000b). Thus, the ability of *C. albicans* to sense and to appropriately respond to environmental pH is critical for pathogenesis.

In *C. albicans*, Rim101p is activated by a number of upstream pathway members, including Rim8p and Rim20p (Porta *et al.*, 1999; Davis *et al.*, 2000a). Rim101p activation occurs by proteolytic removal of an inhibitory C-terminal domain in *Saccharomyces cerevisiae* and *Aspergillus nidulans* (Orejas *et al.*, 1995; Li and Mitchell,

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1997; Diez *et al.*, 2002); genetic and biochemical evidence suggests this is also the case in *C. albicans* (Davis *et al.*, 2000a,b; El Barkani *et al.*, 2000; Porta *et al.*, 2001; Davis, 2003; Li *et al.*, 2004). Rim101p proteolytic processing is governed by extracellular pH: in acidic environments, unprocessed (full-length) Rim101p predominates; in alkaline environments a processed form predominates that promotes changes in gene expression (Denison, 2000). Thus, this signal transduction pathway links extracellular pH to changes in gene expression.

In *S. cerevisiae*, Rim101p is required for growth at alkaline pH and in the presence of high concentrations of lithium (Lamb *et al.*, 2001). In this model fungus, Rim101p functions as a positive activator of *ENA1*, a sodium efflux pump required for growth at alkaline pH and in the presence of high concentrations of salt, including lithium (Lamb *et al.*, 2001). However, Rim101p does not directly regulate *ENA1* expression. At alkaline pH, Rim101p represses *NRG1*, which encodes a Tup1p-dependent transcriptional repressor of *ENA1*. Mutant studies demonstrated that *RIM101* is dispensable for alkaline growth in an *nrg1*- mutant background or when *ENA1* is expressed from a constitutive promoter (Lamb and Mitchell, 2003). Thus, in *S. cerevisiae*, the function of the *RIM101* pathway for adaptation to alkaline pH is to repress *NRG1* expression, thus allowing induction of the Ena1p sodium efflux pump.

In this study we have begun to elucidate the role of the *RIM101* pathway for alkaline pH responses in *C. albicans*. Through mutant studies, we demonstrate that the function of Rim101p does not act through Nrg1p in *C. albicans*. To identify possible functions for the *RIM101* pathway in *C. albicans*, we used whole-genome microarrays to study the transcriptional profile of wild-type and *rim101*- mutant cells grown at acidic and alkaline pH. We found that many genes predicted to function in iron metabolism are pH regulated and that those expressed at a higher level at alkaline pH compared with acidic pH were Rim101p dependent. We also demonstrate that *rim101*- mutant strains were sensitive to iron starvation and thus one important role for the *RIM101* pathway in alkaline environments is to adapt to iron starvation.

Results

Rim101p governs pH responses independent of Nrg1p

In *S. cerevisiae*, Rim101p positively regulates growth at alkaline pH and in the presence of LiCl through repression of *NRG1*, which encodes a transcriptional repressor of a sodium efflux pump (Lamb *et al.*, 2001; Lamb and Mitchell, 2003). Thus, we hypothesized that, by analogy to the situation in *S. cerevisiae*, the growth and morphological phenotypes of the *C. albicans rim101*- mutant may be governed by *NRG1*.

To test this hypothesis, we generated *rim101*- *nrg1*- double mutants and asked whether loss of Nrg1p function rescues the growth phenotypes of the *rim101*- mutant. Homozygous *nrg1*- mutants were generated in a wild-type and *rim101*- background and confirmed by polymerase chain reaction (PCR) using primers flanking the site of integration (Fig. 1A and B) and Southern blot hybridization (Fig. 1C and D). Furthermore, Northern blot analysis showed that *NRG1* was expressed in the parental strains but not the resulting homozygous mutants (Fig. 1E). Thus, we were able to generate an *nrg1*- null allele in both the wild-type and *rim101*- mutant backgrounds.

To determine whether *rim101*- phenotypes are dependent on *NRG1*, wild-type, *rim101*- and *nrg1*- single mutants, and *rim101*- *nrg1*- double mutants were plated on YPD, YPD buffered at pH 9, and YPD + 150 mM LiCl plates and grown at 37°C (Fig. 2). All strains grew well on YPD medium at 37°C (Fig. 2). Furthermore, wild-type and *nrg1*- single mutants grew similarly at pH 9 and on LiCl, suggesting that Nrg1p is not required for growth at alkaline pH or for growth in the presence of high lithium salt concentrations. We noted that *nrg1*- colonies are often smaller than wild-type colonies, which probably reflects the constitutive filamentous growth pattern of *nrg1*- mutants compared with wild-type cells. As expected, *rim101*- single mutants had severe growth defects both at pH 9 and on LiCl media. Finally, we found that *rim101*- *nrg1*- double mutants were constitutively filamentous but still had a severe growth defects similar to the *rim101*- mutant at pH 9 and on LiCl media (Fig. 2). This result suggests that the growth defects associated with loss of Rim101p function are not rescued by the loss of Nrg1p function.

To test whether the growth and morphological defects seen in the *rim101*- *nrg1*- double mutant resulted from loss of either *RIM101* or *NRG1* sequences, we reintroduced wild-type copies of *RIM101* and *NRG1* and analysed these strains for complementation. Introduction of wild-type *RIM101* into the *rim101*- *nrg1*- double mutant, restored growth both at pH 9 and on LiCl media, but did not rescue the constitutive filamentation phenotype. Introduction of wild-type *NRG1* into the *rim101*- *nrg1*- double mutant failed to restore growth either at pH 9 or on LiCl media, but did rescue the constitutive filamentation phenotype. Thus, these results suggest that Nrg1p does not act downstream of Rim101p in *C. albicans* for growth at alkaline pH or on high concentrations of lithium.

RIM101 and NRG1 act in parallel to govern filamentation

Alkaline pH promotes filamentous growth, which is positively regulated by Rim101p and negatively regulated by

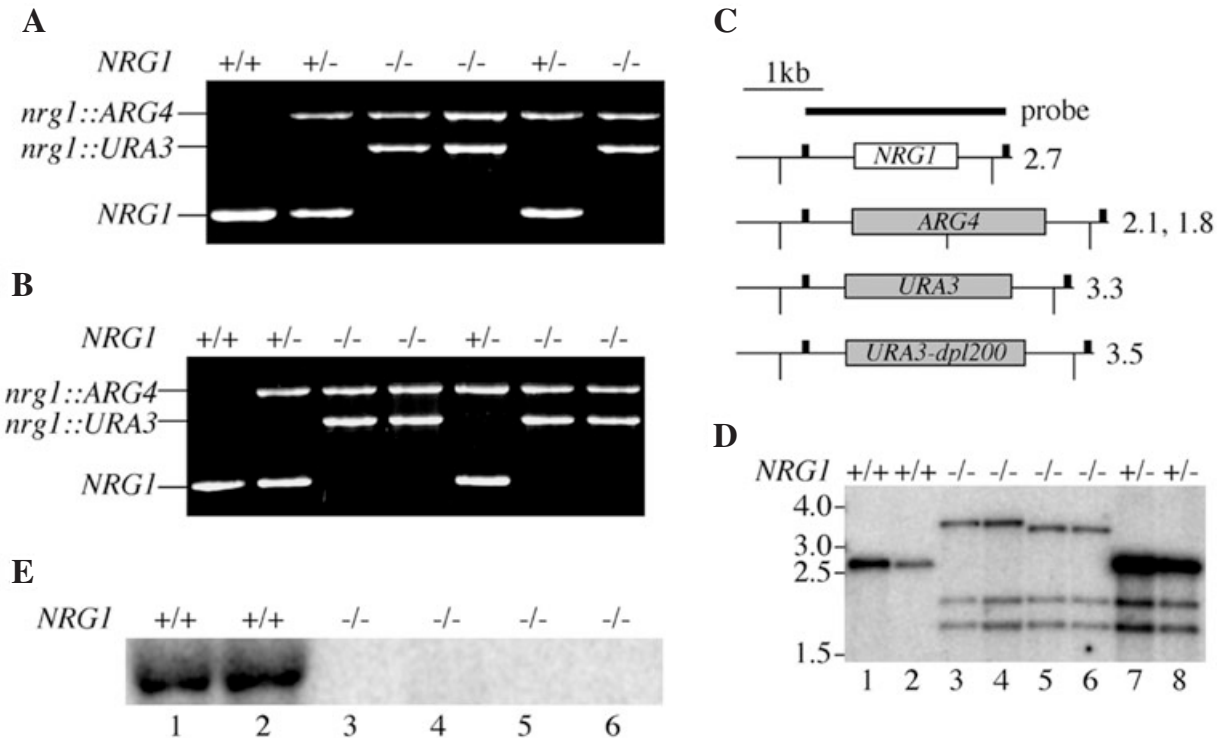


Fig. 1. Confirmation of the *nrg1* deletion.

A and B. PCR analysis of wild-type (A) and *rim101*^{-/-} (B) strains. PCR products were amplified using genomic DNA from (A) BWP17, DAY587, DAY600, DAY601, DAY588 and DAY599 and (B) DAY432, DAY590, DAY593, DAY594, DAY591, DAY595 and DAY596 respectively. C. Diagram of the wild-type *NRG1* locus and deletion alleles. Lines below each allele represent the position of *Bgl*II restriction sites and the numbers to the right of each allele are the predicted sizes (kb) obtained using the *NRG1* probe. Bars above each allele represent the extent of homology to the *NRG1* probe. Note each selectable marker cassette (grey box) replaces the *NRG1* coding sequence (white box). D. Southern blot analysis of *Bgl*II-digested genomic DNA from *NRG1*^{+/+} (BWP17 and DAY432; lanes 1 and 2), *NRG1*^{+/-} (DAY587 and DAY591; lanes 7 and 8) and *nrg1*^{-/-} (DAY607, 603, 600, and 595; lanes 3–6) strains. E. Confirmation of the *nrg1* deletion by Northern blot. RNA was purified from *NRG1*^{+/+} (DAY5 and DAY286; lanes 1 and 2) cells and *nrg1*^{-/-} cells (DAY593, 607, 600 and 603; lanes 3–6) in an otherwise wild-type (DAY286, 600 and 603; lanes 2, 5 and 6) or *rim101*^{-/-} background (DAY5, 593 and 607; lanes 1, 3 and 4). Blots were probed for *NRG1* and then *TEF1* as a loading control (data not shown).

Nrg1p (Ramon *et al.*, 1999; Davis *et al.*, 2000a; Braun *et al.*, 2001; Murad *et al.*, 2001a). On M199 medium buffered at pH 8, wild-type cells formed smooth colonies with filaments at the periphery (Fig. 3). As expected, the *rim101*^{-/-} mutant formed a smooth colony without filaments at the periphery, which is restored to wild-type by introduction of a wild-type copy of *RIM101*; the *nrg1*^{-/-} mutant formed a rough colony with extensive filaments at the periphery. Introduction of a wild-type copy of *NRG1* rescued most of the smooth colony phenotype and reduced filament formation at the periphery to wild-type levels. Finally, the *rim101*^{-/-} *nrg1*^{-/-} double mutant formed rough colonies similar to the *nrg1*^{-/-} colonies. However, like the *rim101*^{-/-} colonies, but unlike the wild-type or *nrg1*^{-/-} colonies, they lacked filaments at the periphery, suggesting that peripheral filamentous growth at pH 8 is dependent on Rim101p. Introduction of a wild-type copy of *RIM101* into the *rim101*^{-/-} *nrg1*^{-/-} double mutant results in a rough colony with extensive filaments at the periphery, like the *nrg1*^{-/-} single mutant; introduc-

tion of a wild-type copy of *NRG1* into the *rim101*^{-/-} *nrg1*^{-/-} double mutant results in a more smooth colony that lacks filaments at the periphery, like the *rim101*^{-/-} single mutant. Thus, at pH 8, the constitutive filamentation phenotype of *nrg1*^{-/-} strains is partially Rim101p dependent. Taken together, our growth and filamentation data suggests that Rim101p does not act through Nrg1p, unlike the case in *S. cerevisiae*.

The pH response in wild-type cells

As the role of Rim101p in *C. albicans* for pH responses appears to be distinct from the role seen in *S. cerevisiae*, we utilized whole-genome microarrays to compare the transcriptional profiles of wild-type and *rim101*^{-/-} mutant cells at acidic (pH 4) and alkaline (pH 8) pH.

We first determined the transcriptional profile of DAY185 prototrophic wild-type cells grown for 4 h at 37°C in M199 medium buffered at pH 4 or pH 8. Under these conditions, 4715 open reading frames (ORFs) gave at

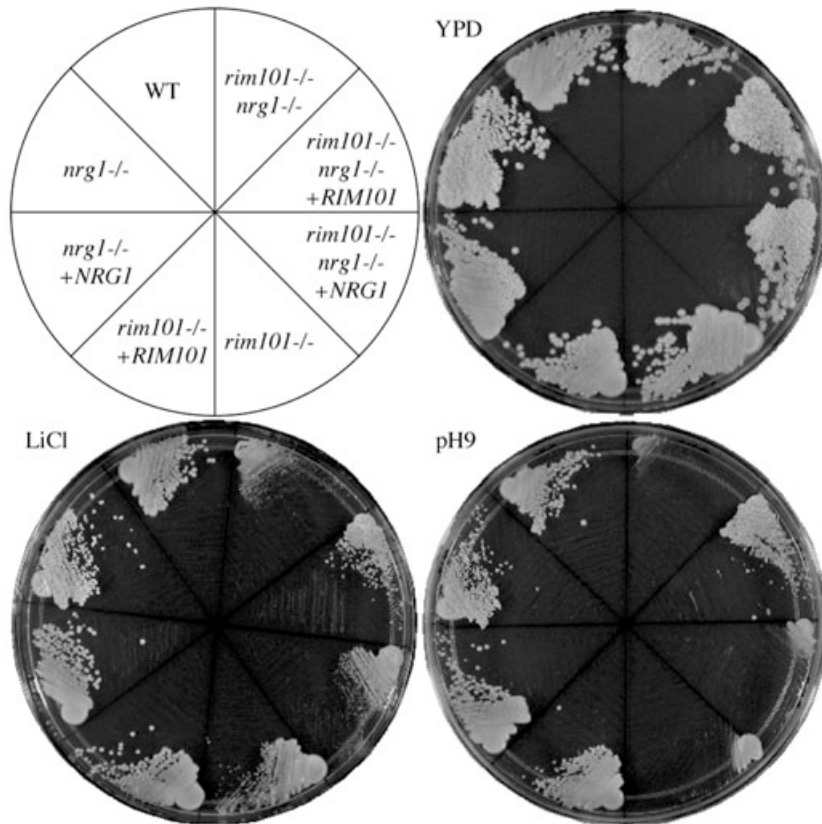


Fig. 2. Growth phenotypes of *nrg1*^{-/-} mutants. Cells from overnight YPD cultures were of wild-type (DAY185), *rim101*^{-/-} (DAY25), *nrg1*^{-/-} (DAY816) and *rim101*^{-/-} *nrg1*^{-/-} (DAY778), and the respective complemented strains DAY463, DAY814, DAY774 and DAY808 were spotted on YPD, YPD pH 9 and YPD + LiCl plates and incubated at 37°C and photographed after 2 days of growth.

least four spots of reliable signal (with an average of 6.8 spots per ORF) and were analysed by SAM 1.21 to identify ORFs that had statistically significant differences between the two conditions (Table 1); and 1084 ORFs showed a statistically significant difference in expression between these two conditions ($\approx 23\%$ of the genes with a reliable number of spots). The remaining ORFs showed no change in expression after 4 h of growth at pH 4 versus pH 8. Of these 1084 ORFs, 514 ORFs had a statistically significant difference of ≥ 2 -fold at either pH 4 or pH 8.

We considered the possibility that some of these 514 ORFs may result from random variation within the data. To determine how often false-positive signals arise, we compared the transcriptional profile of two independent samples of DAY185 prototrophic wild-type cells grown for 4 h at pH 4. As these samples are derived from isogenic strains grown under similar conditions, we expected the transcriptional profiles to be similar. Of 5020 ORFs with reliable signal, only four (0.08%) had a statistically significant difference between the wild-type samples tested and no ORFs had a statistically significant difference of ≥ 2 -fold. Thus, there is a very low level of false-positive results under the conditions being studied. Based on these results, we have used the ≥ 2 -fold difference as our criteria for data analysis.

Of the 514 pH-responsive ORFs, 247 (48%) showed ≥ 2 -fold less expression at pH 8 than at pH 4, which we refer to as alkaline-down-ORFs. The remaining 267 ORFs (52%) showed ≥ 2 -fold more expression at pH 8

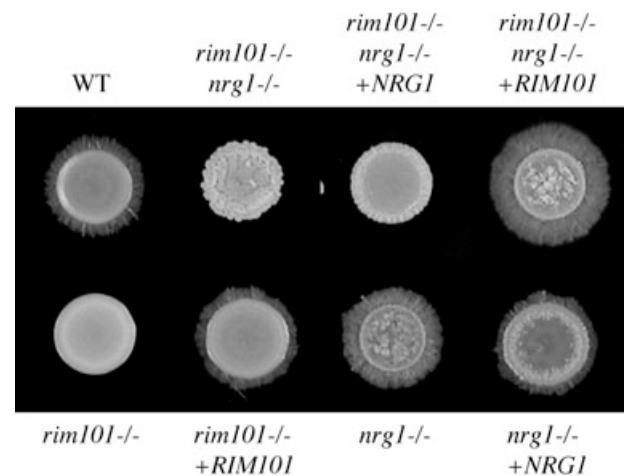


Fig. 3. Alkaline-induced filamentation. Wild-type (DAY185), *rim101*^{-/-} (DAY25), *nrg1*^{-/-} (DAY816), *rim101*^{-/-} *nrg1*^{-/-} (DAY778) and the respective complemented strains (DAY463, DAY814, DAY774 and DAY808) were spotted onto M199 buffered at pH 8 and incubated at 37°C for 5 days before photography.

Table 1. Microarray experiment results.

Condition	No. of slides	No. of ORFs analysed	Average No. of valid spots per ORF (minimum = 4)	No. of differentially expressed (SAM)	No. of ≥ 2 -fold difference
WT pH 4 versus WT pH 8	4	4715	6.8	1084	514
WT pH 4 versus <i>rim101</i> ^{-/-} pH 4	6	5019	8.2	87	8
WT pH 8 versus <i>rim101</i> ^{-/-} pH 8	5	5404	8.2	487	186
WT pH 4 versus WT pH 4	4	5020	6.9	4	0

than at pH 4, which we refer to as alkaline-up-ORFs. We utilized the alkaline-up/alkaline-down designations, because they provide a better framework for understanding the results obtained with the *rim101*^{-/-} mutant (see below).

Previous studies have identified several pH-responsive genes (Davis, 2003). We analysed our microarray data to determine whether these genes were also identified in the collection of 514 pH-responsive ORFs. *PHR2* and *RIM8*, which encode a cell wall protein and a member of the *RIM101* pH response pathway, are expressed preferentially in acidic environments compared with alkaline environments (Muhlschlegel and Fonzi, 1997; Porta *et al.*, 1999). On the microarrays, these genes were expressed at 10-fold and 2.6-fold lower levels at pH 8 compared with pH 4 respectively (Table 2). Furthermore, *PHR1* and *PRA1*, which encode two cell wall proteins, and *RIM101* are preferentially expressed at alkaline pH compared with acidic pH (Saporito-Irwin *et al.*, 1995; Sentandreu *et al.*, 1998; Ramon *et al.*, 1999; Davis *et al.*, 2000a). On the microarrays, these genes were expressed at 60-, 278- and threefold more at pH 8 compared with pH 4 respectively. Finally, growth at 37°C and pH 8 promotes hyphal cell growth and growth at 37°C and pH 4 promotes yeast cell growth. Consistent with this, one class of alkaline-up-ORFs was predicted to encode hyphal-specific genes. Indeed, the known hyphal-specific genes (*CSA1/WAP1*, *ECE1*, *HWP1*, *HYR1*, *IHD1*, *RBT1*, *SAP4* and *SAP6*) were expressed two- to 31-fold more at pH 8 compared with pH 4 (Tables 3 and 4). Thus, the *C. albicans* whole-genome microarray identified alkaline-down-ORFs and alkaline-up-ORFs previously identified by other

approaches, suggesting that this approach can also be used to identify new pH-responsive ORFs.

In order to identify processes that may be sensitive to acidic or alkaline pH, the pH-responsive ORFs were classified according to predicted gene function using Gene Ontology (GO) categories (Table 3). For the alkaline-down-ORFs, 45% either had no homologues (novel) or had a homologue, usually in *S. cerevisiae*, of unknown function (unknown). For the alkaline-up-ORFs, 30% either had no homologues (novel) or had a homologue, usually in *S. cerevisiae*, of unknown function (unknown). We found a pH-dependent expression bias in the categories of genes involved in hyphal growth (described above), ion transport, protein synthesis/localization and electron transport (Table 3).

Genes predicted to function in ion transport were expressed at higher levels more often at pH 8 than at pH 4. Of the 17 alkaline-up-ORFs classified in the ion transport class (Table 4), 10 are predicted to function in iron transport. These include two high-affinity iron permeases (*FTR1/2* and *FTH1*), one multicopper oxidase (*FET34*), five ferric reductases (*CFL1*, *FRE2*, *FRE7*, *FRE9* and *FRP2*), one vacuolar iron transporter (*SMF3*) and one iron-siderophore binding protein (*ARN1/SIT1*). The remaining alkaline-up-ORFs in this class are predicted to encode transporters for phosphate (*PHO84* and *PHO89*), copper (*CTR1*), manganese (*SMF1*), sodium (*ENA2*), zinc (*ZRT1*) and an unknown ion (6.6692). In contrast, of the seven alkaline-down-ORFs classified in the ion transport class (Table 2), only two are predicted to function in iron transport, *RBT2* and 6.5606, which encode putative ferric reductases. An additional alkaline-down-ORF, *ISA1*,

Table 2. Alkaline-down-ORFs in particular Gene Ontology (GO) categories.

Amino acid metabolism	<i>ACO1</i> (5837), <i>ARG1</i> (8721), <i>ARG3</i> (5062), <i>ARG4</i> (6798), <i>ARG5,6</i> (4898), <i>ARO10</i> (5614), <i>CHA1</i> (6363), <i>CPA1</i> (2053), <i>GAD1</i> (3128) <i>GLT1</i> (2272), <i>GLY1</i> (5812,1595), <i>LEU1</i> (8750)
Ion transport	<i>CCC1</i> (7334), <i>CCC2</i> (4894), <i>ISA1</i> (4463), <i>PHO87</i> (1540), <i>RBT2</i> (6384), <i>ZRT2</i> (5335), 6.5606
Antioxidants	<i>AHP1</i> (6590), <i>CCP1</i> (957), <i>CTA1</i> (5127), <i>DOT5</i> (8653), <i>SOD2</i> (4731), <i>TTR1</i> (7603)
Signal transduction/ transcription factors	<i>CRZ2</i> (1328,2759), <i>FCR1</i> (8814), <i>NRG1</i> (8050), <i>RIM8</i> (6250)
Transporters	<i>CDR1</i> (1644), <i>CDR3</i> (3101), <i>CDR4</i> (6297), <i>DUR3</i> (6765), <i>GAP1</i> (7739), <i>GIT1</i> (2481), <i>GNP1</i> (2846), <i>HXT5</i> (6321), <i>ITR1</i> (8683), <i>JEN1</i> (8607,8296), <i>OPT1</i> (2195), <i>STL1</i> (8460), <i>YOR049C</i> (1289), 6.3118, 6.5454 , 6.7323
Cell wall	<i>CHT1</i> (8769), <i>PHR2</i> (6260), <i>ROT2</i> (649), <i>SCW4</i> (4005), <i>YDL225C</i> (1074), <i>YDR533C</i> (1991), 6.4236, 6.8118
Electron transport	<i>CYB2</i> (8009), <i>CYC1</i> (244), <i>CYT1</i> (2040), <i>MCR1</i> (4367), <i>RIP1</i> (9144), <i>YOR256W</i> (1483), 6.4187, 6.4597, 6.7629, 6.8837

Boldface denotes ORFs expressed ≥ 5 -fold. Number in parentheses is the ORF6 designation(s).

Table 3. Gene Ontology (GO) categories.

	Alkaline-down	Alkaline-up	<i>rim101</i> -up	<i>rim101</i> -down	RIM101-pH-independent
Amino acid metabolism	13	19	1	4	2
Carbohydrate metabolism	11	15	3	4	4
Lipid metabolism	6	11	1	4	5
Other metabolism	24	13	2	9	3
Glyoxylate pathway	2	2	0	5	4
Antioxidant	6	2	1	2	0
Ion transport	7	17	5	11	3
Other transport	17	13	4	14	7
Signal transduction/transcription	5	8	2	1	2
Protein synthesis/local	6	42	2	3	3
Protein folding/degradation	8	1	0	0	1
Peroxisomal	2	3	0	0	0
Electron transport	11	0	0	0	0
Hyphal	0	11	0	7	0
Cell wall	8	14	2	10	3
Other	10	16	4	9	5
Unknown	43	35	5	9	6
Novel ^a	68	45	23	39	22
Total	247	267	55	131	70

a. Novel ORFs were defined as having no homologue $<e^{-10}$ by BLAST analysis.

encodes a protein predicted to function in transport of iron from the cytoplasm into the mitochondria. The remaining alkaline-down-ORFs in this class encode predicted transporters for phosphate (*PHO87*), manganese (*CCC1*), zinc (*ZRT2*) and copper (*CCC2*). It should be noted that *Ccc2p* is indirectly required for high-affinity iron uptake in *C. albicans*, because copper is a cofactor for the multicopper oxidase (Weissman *et al.*, 2002).

Another class of ORFs with increased expression at pH 8 versus pH 4 is the protein synthesis/localization class. This class contains 42 alkaline-up-ORFs, including 29 ORFs for ribosomal biosynthesis, three ORFs required for protein glycosylation and 10 ORFs important for protein trafficking. While an increase in the expression of genes involved in protein synthesis could reflect a difference in growth rate, this is unlikely because *C. albicans* grows slower at pH 8 than at pH 4 (Davis *et al.*, 2000a).

Ten alkaline-down-ORFs encode proteins important for the synthesis of electron transport complexes. Six of these encode homologues of *S. cerevisiae* *MCR1*, *CYB2*, *CYT1*, *RIP1*, *CYC1* and *YOR356W*, three encode homologues of *Yarrowia lipolytica* complex I (NADH:ubiquinone oxidoreductase) subunits, and one encodes a homologue of *C. tropicalis* NADH dehydrogenase complex I. These *C. albicans* homologues had BLAST *E*-values of $\leq e^{-73}$. Also, three alkaline-down-ORFs in the 'other metabolism' category encode proteins involved in haem biosynthesis (*HEM3*, *HEM4* and *COX15*). These haem biosynthetic genes may be co-regulated with the electron transport ORFs.

The pH response in *rim101*–/– mutant cells

To determine how the *RIM101* pathway contributes to the pH response, we compared the transcriptional response

Table 4. Alkaline-up-ORFs in particular Gene Ontology (GO) categories.

Amino acid metabolism	<i>AAT1</i> (4647), <i>ARO4</i> (3017), <i>ARO7</i> (3439), <i>ARO8</i> (7457), <i>CAR1</i>(2297) , <i>CAR2</i>(4510) , <i>CYS3</i> (7852), <i>CYS4</i> (6682), <i>IDP1</i> (3902), <i>ILV6</i> (3347), <i>LEU3</i>(484) <i>MET25</i> (4514), <i>PRO3</i>(4519,6890) , <i>PUT1</i> (3483), <i>PUT2</i> (5650), <i>SAH1</i> (5137), <i>SAM2</i> (2190), <i>SAM4</i>(6278)
Ion transport	<i>ARN1</i>(6084) , <i>CFL1</i> (5730), <i>CTR1</i>(3031) , <i>ENA2</i>(7614) , <i>FET34</i> (6.2939), <i>FRE2</i>(6.5731) , <i>FRE7</i> (5675), <i>FRE9</i> (4284), <i>FRP2</i>(8441) , <i>FTH1</i> (3061), <i>FTR1/2</i> (8131), ^a <i>PHO84</i> (3437), <i>PHO89</i>(7898) , <i>SMF1</i>(3571) , <i>SMF3</i> (7486), <i>ZRT1</i>(6933) , 6.6692
Antioxidants	<i>GPX2</i> (1558), <i>SOD1</i> (7493)
Signal transduction/ Transcription factors	<i>AAF1</i> (8596), <i>GPR1</i> (5595), <i>HAP3</i> (3344), <i>PTP3</i> (8958), <i>RGD1</i> (3621), <i>RIM101</i> (8147), <i>TEC1</i> (9129), <i>UME6</i>(1326)
Transporters	<i>GAP1</i> (713, 1878), <i>GAP1</i>(4609,6768) , <i>GNP1</i> (8914), <i>HIP1</i> (4405), <i>MAL31</i> (5657), <i>MUP1</i> (7662), <i>SFC1</i> (1915), <i>YDL199C</i> (3782), <i>YHM2</i> (2738), <i>YOL119C</i> (2533), <i>YPR021C</i> (2839)
Cell wall	<i>ALS3/8</i> , <i>CHT2</i> (2344), <i>CRH1</i> (1231), <i>ECM38</i> (4887), <i>KRE6</i> (8523), <i>PHR1</i>(7524) , <i>PRA1</i>(6934) , <i>PSA1</i> (5411), <i>YDL103</i> (3492), <i>YDL219W</i> (4808), 6.857 , 6.1115 , 6.1377 , 6.3331
Hyphal	<i>CSA1/WAP1</i>(8439) , <i>ECE1</i>(2886) , <i>HWP1</i>(4883) , <i>HYR1</i>(3143) , <i>IHD1</i>(6198) , <i>RBT1</i>(4889) , <i>SAP4</i>(10026) , <i>SAP6</i> (10028), <i>MP65</i> (708), <i>YPL184</i> (2205), <i>RBT4</i> (5422)

a. The primer set used does not distinguish *FTR1* and *FTR2*, thus these two genes have been grouped together. Boldface denotes ORFs expressed ≥ 5 -fold. Number in parentheses is the ORF6 designation(s).

Table 5. Rim101p-dependent genes in particular Gene Ontology (GO) categories.

Rim101p-dependent genes at pH 4	
<i>rim101</i> ^{-/-} pH 4 Up	6.450 , ^a 6.6747 ^a
<i>rim101</i> ^{-/-} pH 4 Down	<i>ASN1</i> (209), <i>GCV2</i> (6279), ^a <i>HIS1(7439)</i> , ^a <i>PRE1(7438)</i> , ^a <i>RBT5</i> (4505), ^a 6.6926 ^a
<i>rim101</i> -up-ORFs at pH 8	
Ion transport	<i>FRE3</i> (5606), ^b <i>PHO87</i> (1540), ^b <i>RBT2</i> (6384) ^b
Antioxidants	<i>CTA1</i> (5127) ^b
Signal transduction/ transcription factors	<i>CRZ2(1328,2759)</i> ^b
Transporters	<i>GNP1</i> (2846), ^b <i>JEN1(8296)</i> , ^b <i>MDR1</i> (5068), 6.5454 , ^b 6.7323 ^b
Cell wall	<i>PHR2(6260)</i> , ^b <i>YDL222C</i> (1074) ^b
<i>rim101</i> -down-ORFs at pH 8	
Amino acid metabolism	<i>ARG1</i> (8721), <i>GDH3</i> (3152), <i>HIS1(7439)</i> , <i>PUT1</i> (3483) ^c
Ion transport	<i>ARN1</i> (6084), ^c <i>CTR1(3031)</i> , ^c <i>ENA2</i> (7614), ^c <i>FET34</i> (6.2939), ^c <i>FRE2</i> (6.5731), ^c <i>FRE5(4503)</i> , <i>FRP2(8441)</i> , ^c <i>PHO89</i> (7898), ^c <i>YLR205C</i> (7617), <i>ZRT1(6933)</i> , ^c 6.6692 ^c
Antioxidants	<i>GPX2</i> (1558), ^c <i>SOD1</i> (7493) ^c
Signal transduction/ transcription factors	<i>RIM101</i> (8147) ^c
Transporters	<i>CDR1</i> (1644,9037), ^c <i>DIP5</i> (4006,4007), <i>FUN34</i> (6177,6465), <i>GAP1</i> (4609), ^c <i>GNP1</i> (8914), <i>MUP1</i> (7662), ^c <i>PDR10</i> (1495), <i>RGT2</i> (6479), <i>YOL119C</i> (2533), ^c 6.4217
Cell wall	<i>CRH1</i> (1231), ^c <i>KRE6</i> (8523), ^c <i>PHR1(7524)</i> , ^c <i>PRA1(6934)</i> , ^c 6.857, ^c 6.1115 ^c , 6.1377 , ^c 6.3288, 6.3331, ^c 6.4504
Hyphal	<i>CSA1/WAP1(8439)</i> , ^c <i>ECE1(2886)</i> , ^c <i>HWP1(4883)</i> , ^c <i>HYR1(3143)</i> , ^c <i>IHD1(6198)</i> , ^c <i>RBT1(4889)</i> , ^c 6.6914

a. ORFs also differentially expressed ≥ 2 -fold at pH 8.

b. Also alkaline-down-ORFs.

c. Also alkaline-up-ORFs.

Boldface denotes ORFs expressed ≥ 5 -fold. Number in parentheses is the ORF6 designation(s).

of a *rim101*^{-/-} mutant (DAY25) with that of the wild-type strain (DAY185) at pH 4 and pH 8. At pH 4, of the 5019 ORFs with reliable signal only eight had a ≥ 2 -fold difference in *rim101*^{-/-} cells compared with wild-type cells (Tables 1 and 5). Of these, only one ORF appeared to be specifically expressed ≥ 2 -fold at pH 4 in *rim101*^{-/-} mutant cells compared with wild-type cells (Fig. 4). This ORF is *ASN1*, which encodes asparagine synthase (Dang *et al.*, 1996). The other seven ORFs also showed a ≥ 2 -fold difference in *rim101*^{-/-} cells compared with wild-type cells at pH 8. Thus, in *C. albicans*, Rim101p appears to be dispensable for the transcriptional response to acidic conditions.

At pH 8, of the 5404 ORFs with reliable signal, 186 ORFs showed a statistically significant difference of ≥ 2 -fold in the *rim101*^{-/-} mutant compared with wild type (Table 1). This group of 186 ORFs were further classified into three groups: 49 ORFs (26%) were also alkaline-down-ORFs, 67 ORFs (36%) were also alkaline-up-ORFs and 70 ORFs (38%) were not pH regulated in wild-type cells (Fig. 4). These results suggest that Rim101p regulates many pH-responsive genes. Furthermore, in addition to its role in alkaline responses, Rim101p is a positive regulator of growth in other conditions, including in the presence of high concentrations of lithium (Davis *et al.*, 2002). Thus, some of the 70 Rim101p-dependent pH-independent ORFs may encode proteins important for these other responses.

Rim101p is a negative regulator of acidic responses at alkaline pH (Davis, 2003). Thus, we predicted that some alkaline-down-ORFs would also be up in the *rim101*^{-/-}

mutant compared with wild-type at pH 8. For example, *PHR2* is an acidic response gene that is repressed at alkaline pH by Rim101p (Muhlschlegel and Fonzi, 1997; Porta *et al.*, 1999; Ramon *et al.*, 1999; Davis *et al.*, 2000a). On the microarrays, *PHR2* was identified as an alkaline-down-ORF and as a *rim101*-up-ORF (Tables 2 and 5). An additional 28 alkaline-down-ORFs were also

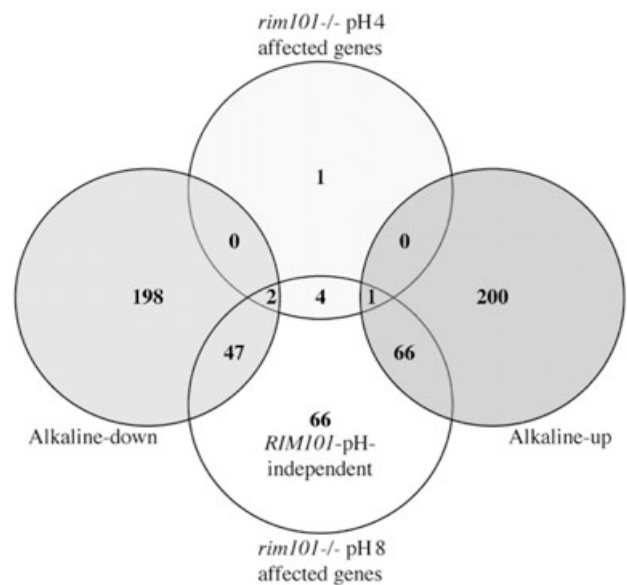


Fig. 4. VENN diagram of microarray results. Representation of the alkaline-down-ORFs (genes up at pH 4) and alkaline-up-ORFs (genes up at pH 8) from wild-type cells and pH 4 affected ORFs and pH 8 affected ORFs from *rim101*^{-/-} cells. Rim101p-dependent ORFs that are pH independent are noted.

rim101-up-ORFs. These alkaline-down-, *rim101*-up-ORFs were found in the same GO categories as the alkaline-down-ORFs except for the 'protein folding/degradation' and 'electron transport' categories (Table 3). This suggests that Rim101p negatively regulates a subset of acidic response genes at alkaline pH.

Rim101p is also a positive regulator of alkaline responses at alkaline pH (Davis, 2003). Thus, we predicted that some alkaline-up-ORFs would be down in the *rim101*^{-/-} mutant compared with wild-type at pH 8. For example, *PHR1* and *PRA1* are alkaline response genes that are induced at alkaline pH by Rim101p (Saporito-Irwin *et al.*, 1995; Sentandreu *et al.*, 1998; Porta *et al.*, 1999; Ramon *et al.*, 1999; Davis *et al.*, 2000a). On the microarrays, *PHR1* and *PRA1* were identified at alkaline-up-ORFs and *rim101*-down-ORFs (Tables 2 and 5). An additional 62 alkaline-up-ORFs were also *rim101*-down-ORFs. These alkaline-up-, *rim101*-down-ORFs were found in all of the same GO categories as the alkaline-up-ORFs, except for the 'protein synthesis/localization' category (Table 3). This suggests that Rim101p positively regulates specific aspects of the alkaline response, including hyphal formation and ion transport, but that it is dispensable for other aspects of the alkaline response.

Northern analysis of gene expression

To corroborate the microarray data, we analysed a number of pH-responsive targets identified in our microarray studies on Northern blots. We found that *PRA1*, a known pH- and Rim101p-dependent target, and the hyphal-specific genes *ECE1*, *HWP1* and *RBT5* identified as alkaline-up-, *rim101*-down-ORFs on the arrays were expressed at higher levels at pH 8 than at pH 4 in wild-type cells and were not induced in *rim101*^{-/-} cells (Fig. 5). In addition, 6.2929, which encodes a protein with homology to Hwp1p and Rbt1p, was identified as an alkaline-up-ORF and a *rim101*-down-ORF both on the microarrays and on Northern blots. Furthermore, expression of three genes predicted to function in iron transport (*FRP2*, *FRE2* and *ARN1*), zinc transport (*ZRT1*) and sodium efflux (*ENA2*) was increased ≥ 10 -fold at pH 8 compared with pH 4 in wild-type cells and this increase was Rim101p-dependent (Fig. 5). Thus, many genes required for ion transport are induced under alkaline conditions and this induction requires Rim101p.

Northern blot analysis also confirmed our identification of alkaline-down-, *rim101*-up-ORFs. In wild-type cells, 6.5454 and *CRZ2* were expressed at higher levels at pH 4 than at pH 8 (Fig. 5) and expression of these genes was similar in both wild-type and *rim101*^{-/-} cells at pH 4. However, expression of *CRZ2* and 6.5454 was higher in *rim101*^{-/-} cells at pH 8 than it was in either wild-type or *rim101*^{-/-} cells at pH 4. Thus, *CRZ2* and 6.5454 require

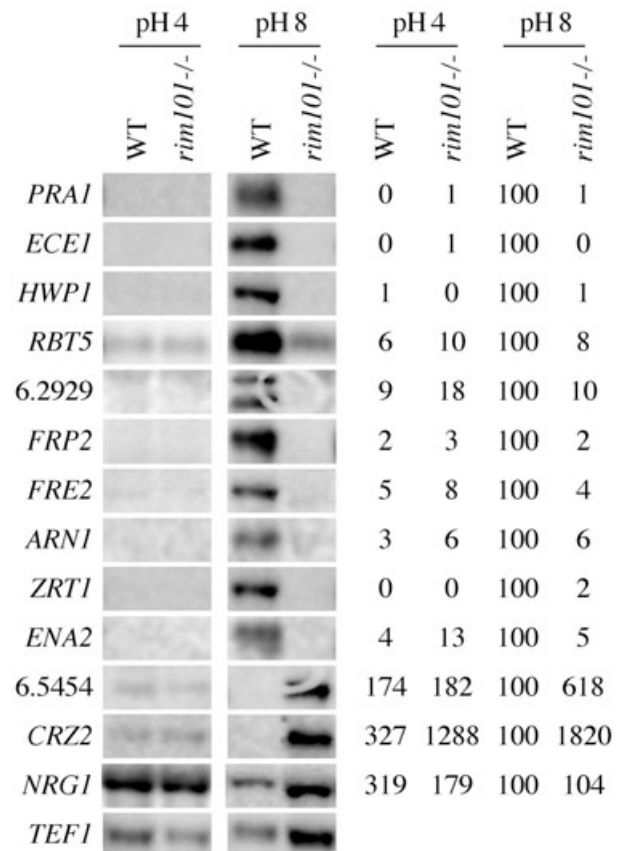


Fig. 5. Northern blot analysis of selected ORFs. RNA was purified from wild-type (DAY185) and *rim101*^{-/-} (DAY25) cells, separated by gel electrophoresis and hybridized to ORFs listed on the left. Numbers on the right represent the signal quantified with a phosphorimager, normalized for *TEF1* expression, which serves as a loading control, and expressed as a percentage of the wild-type pH 8 signal.

Rim101p for repression at pH 8. Furthermore, in the absence of Rim101p, these alkaline-down-ORFs become alkaline-up-ORFs. These results are similar to those reported previously for *PHR2* and support the model that a Rim101p-independent mechanism governs the expression of some alkaline response genes (Davis *et al.*, 2000a; 2002).

Finally, we analysed the expression of *NRG1*, which by microarray was an alkaline-down-ORF but not differentially expressed in *rim101*^{-/-} cells (Table 2). In wild-type cells, *NRG1* was expressed at threefold higher levels at pH 4 compared with pH 8 (Fig. 5). In *rim101*^{-/-} cells, *NRG1* was also expressed at higher levels at pH 4 compared with pH 8. We noted that the expression in *rim101*^{-/-} cells was slightly reduced compared with wild-type cells at pH 4; however, expression in wild-type and *rim101*^{-/-} cells at pH 8 was indistinguishable. These results support the phenotypic data and supports the hypothesis that the function of Rim101p in pH responses is not through repression of *NRG1* in *C. albicans*.

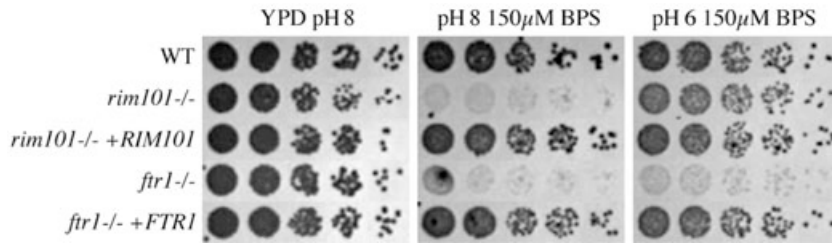


Fig. 6. Growth of *C. albicans* strains in the presence of BPS. Fivefold serial dilutions of wild-type (DAY185), *rim101*^{-/-} (DAY25), *rim101*^{-/-} + *RIM101* (DAY463), *ftr1*^{-/-} (DAY750) and *ftr1*^{-/-} + *FTR1* (DAY749) cells were spotted onto YPD pH 8, YPD pH 8 + 150 μM BPS and YPD pH 6 + 100 μM BPS incubated at 30°C for 2–3 days.

Rim101p is required for growth in iron-limited media at alkaline pH

As many pH- and Rim101p-dependent ORFs appear to function in iron transport, we hypothesized that Rim101p is important for iron metabolism in alkaline conditions. To test this hypothesis, we first analysed the growth of wild-type and *rim101*^{-/-} strains in the presence of the iron chelator bathophenanthroline disulphonate (BPS) (Fig. 6). On YPD medium buffered at pH 8, all strains grew well including the *rim101*^{-/-} mutant. On YPD + 150 μM BPS buffered at pH 8, wild-type cells grew slightly slower than on YPD pH 8 alone; however, individual cells still gave rise to colonies. In contrast, the *ftr1*^{-/-} mutant, which is deleted for the iron starvation-induced high-affinity Fe-transporter, did not grow on this medium. Like the *ftr1*^{-/-} mutant, the *rim101*^{-/-} mutant also had a severe growth defect on YPD + 150 μM BPS buffered at pH 8. Introduction of a wild-type copy of *FTR1* or *RIM101* into the respective mutant completely rescued the growth defect.

As *C. albicans* Rim101p is activated by proteolytic processing in response to alkaline pH (Li *et al.*, 2004), we next asked whether Rim101p is required for growth at a more acidic pH. On YPD + 150 μM BPS buffered at pH 6, we found that the *ftr1*^{-/-} mutant failed to grow, thus the medium is indeed iron limited. However, unlike the situation at pH 8, the *rim101*^{-/-} mutant grew like wild type on YPD + 150 μM BPS buffered at pH 6. Similar results were seen using the divalent cation chelator EDTA and the ferric iron chelator 2'2'-bipyridyl (data not shown). Thus, these results suggest that the role of Rim101p in iron-limited environments is pH dependent.

Although BPS is a strong chelator for iron, it is possible that the *rim101*^{-/-} mutant is not sensitive to iron starvation, but another divalent cation that is chelated by BPS. To address this possibility, we grew cells in iron-depleted media in the absence of chelators. We first grew wild type, *rim101*^{-/-}, *ftr1*^{-/-} and the complemented strains in SD-Fe-Cu medium + 10 nM FeCl₃ buffered at pH 4 (Table 6). All strains, except for the *ftr1*^{-/-} mutant, grew well in this medium compared with wild-type. The high-affinity iron transporter mutant, *ftr1*^{-/-}, did not grow. We next grew cells in SD-Fe-Cu medium + 200 μM FeCl₃ buffered at pH 8 (Table 6). As expected, more FeCl₃ had to be added back to the iron-limited medium at pH 8 compared with

pH 4 to support growth of wild-type cells. In this medium, wild-type cells were able to grow, whereas the *rim101*^{-/-} and the *ftr1*^{-/-} mutants failed to grow over 4 days. Introduction of the *RIM101* and *FTR1* wild-type genes restored growth; however, we noted that the complemented *RIM101* strain only partially rescued the growth defect similar to results described in Fig. 2. Taken together, these results suggest that adaptation to alkaline environments partly results from adaptation to iron starvation and this response is Rim101p dependent.

Discussion

As both a commensal and a pathogen, *C. albicans* must adapt to, and thrive in, a broad range of environmental pH. Using microarrays, we identified more than 500 genes that are differentially regulated in response to pH. In addition, our studies suggest that Rim101p is required for a subset of alkaline responses, consistent with the idea that Rim101p-independent pathways also contribute to adaptation to environmental pH (Davis *et al.*, 2000a; 2002).

The role of Rim101p in the pH response

Microarray analysis of the *rim101*^{-/-} mutant revealed that Rim101p plays an important role in the transcriptional response to alkaline pH. At pH 4, Rim101p specifically affects the transcript levels of only one ORF, suggesting that Rim101p has no significant role in the acidic response. This is consistent with the fact that *rim101*^{-/-} mutant strains grow like wild type in acidic environments

Table 6. Growth of *C. albicans* strains in iron-depleted medium.

Strain	Relevant genotype	Growth ratio compared with WT ^a	
		pH 4	pH 8
DAY185	WT	1.0	1.0
DAY25	<i>rim101</i> ^{-/-}	0.97	NG
DAY44	<i>rim101</i> ^{-/-} + <i>RIM101</i>	0.90	4.9
DAY749	<i>ftr1</i> ^{-/-} + <i>FTR1</i>	0.94	1.6
DAY750	<i>ftr1</i> ^{-/-}	NG	NG

a. Growth is the ratio of wild-type generation time/strain of interest generation time. Data represent the average of three independent samples; standard deviation <15%. NG, no observable growth.

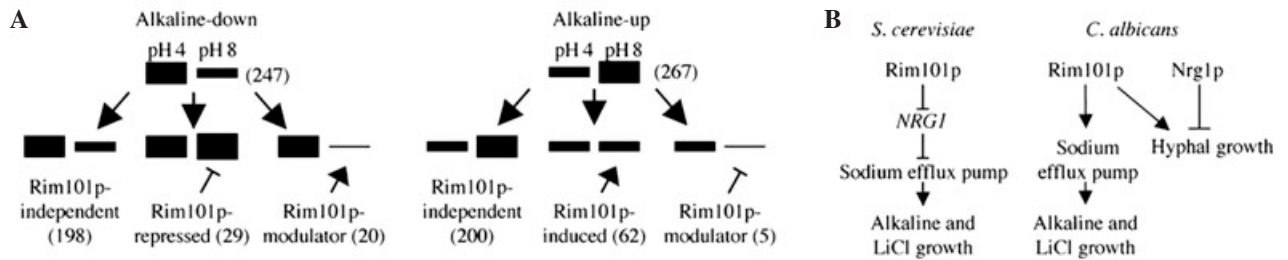


Fig. 7. Models of Rim101p function in *C. albicans*.

A. Cartoon summarizing types of gene expression changes seen in wild-type and *rim101*^{-/-} strains based on the microarray analyses. In wild-type cells, we see genes expressed more at acidic pH than at alkaline pH, alkaline-down, and gene expressed more at alkaline pH than at acidic pH, alkaline-up. In both classes, many genes are not affected by loss of Rim101p, Rim101p-independent. We found alkaline-down-ORFs that require Rim101p for repression, Rim101p-repressed, and alkaline-up-ORFs that require Rim101p for induction, Rim101p-induced. Furthermore, in both the alkaline-down and alkaline-up classes we found a Rim101p-modulator group, in which Rim101p appears to act as an inducer and a repressor respectively. One possible explanation for the Rim101p-modulator group is that additional factors co-regulate these ORFs in conjunction with Rim101p. Numbers in parentheses represent the number of ORFs found in each class.

B. In *S. cerevisiae*, Rim101p and Nrg1p act in a dependent pathway to govern expression of Na⁺-ATPase *ENA1*, which is required for growth in alkaline and LiCl environments. In *C. albicans*, Rim101p governs expression of the Na⁺-ATPase *ENA2*; however, growth in alkaline and LiCl environments is not dependent on Nrg1p. Furthermore, Rim101p and Nrg1p make independent contributions to hyphal formation, which suggests that in *C. albicans*, Rim101p and Nrg1p act in independent pathways.

(Table 6). At pH 8, Rim101p affects the transcript levels of 186 genes, functioning as a repressor of a subset of acidic response genes and as an inducer of a subset of alkaline response genes (Table 5). These data suggest that Rim101p has an important role in the alkaline response and are consistent with the growth and morphological phenotypes of *rim101*^{-/-} strains in alkaline environments.

How does Rim101p contribute to the transcriptional changes seen in response to extracellular pH? We categorized genes, which are expressed in a pH-dependent manner, as either alkaline-down or alkaline-up. In our transcriptional profiling assays of the *rim101*^{-/-} mutant, we found that Rim101p plays a role in both classes (Fig. 7A). Of the alkaline-down-ORFs, 80% are not affected by Rim101p, 12% appear to be repressed by Rim101p at pH 8, including *PHR2*, and 8% are actually expressed at a lower levels in the *rim101*^{-/-} mutant than in wild-type cells at pH 8. One explanation for this latter class is that these ORFs require Rim101p for basal levels of expression at pH 8 and are induced by other factors at pH 4. Of the alkaline-up-ORFs, 75% are not affected by Rim101p, 23% appear to be induced by Rim101p at pH 8, including *PRA1*, and 2% are actually expressed at a lower levels in the *rim101*^{-/-} mutant than in wild-type cells at pH 8. Again, this latter class may represent ORFs that require Rim101p for expression in conjunction with an additional factor and in the absence of Rim101p, this additional factor acts as a repressor. In both the alkaline-down- and alkaline-up-ORFs, we found a group that suggests Rim101p may act in concert with an addition factor(s), thus we have termed these group as Rim101p modulated.

How does Rim101p regulate alkaline pH-dependent gene expression responses? One possibility is that Rim101p binds directly to the promoters of Rim101p-

dependent genes to govern expression. Support for this model comes from MEME analysis (Bailey and Elkan, 1994) of the promoters of 20 alkaline-up-ORFs that were Rim101p dependent, including *PRA1* and *PHR1*. This analysis revealed three putative regulatory motifs, one of which is (G/A)CCAAGAA (Table 7). The (G/A)CCAAGAA motif contains the Rim101p/PacC binding site defined in *S. cerevisiae* and *A. nidulans*, GCCAAG (Tilburn *et al.*, 1995; Espeso *et al.*, 1997; Lamb *et al.*, 2001), and the Rim101p binding site recently defined for the *C. albicans* *PHR1* promoter, CCAAGA (Ramon and Fonzi, 2003). Of the 20 promoters analysed, 14 contain the (G/A)CCAAGAA motif. Furthermore, MEME analysis of 20 alkaline-up-ORFs that are Rim101p independent did not identify the (G/A)CCAAGAA motif. Thus, the promoters of many alkaline pH- and Rim101p-dependent ORFs have conserved sequences, one of which is the Rim101p binding site from other fungi.

Rim101p affects the expression of 23% of the pH response genes (Fig. 4). The remaining pH response

Table 7. Potential regulatory sites in pH-responsive genes.

Alkaline-up promoter	Sequence	No. of sites
<i>MAL31</i> , <i>SHA3</i> , 6.1377, 6.2929, 6.6926, 6.8127	GCCAAG	1
<i>FRP2</i> ^a , <i>PRA1</i> , <i>ZRT1</i> , <i>FRE2</i>	GCCAAG	2
<i>HYR1</i> ^a	GCCAAG	3
<i>CSA1</i> , <i>CTR1</i> , <i>ENA2</i> , 6.3932, <i>FRE2</i>	CCAAGA	1
<i>PHR1</i> , 5.5146, 6.8127	CCAAGA	≥2
<i>ARN1</i> , <i>ECE1</i> , <i>HWP1</i> , <i>MAL1</i> , <i>RBT1</i> , <i>SFC1</i> , 6.4795	GCCAAG or CCAAGA	0

a. One GCCAAG site is not also a CCAAGA site.

genes, including those involved in electron transport and protein synthesis, are Rim101p independent (Table 3). Mds3p, a Rim101p-independent regulator of the *C. albicans* alkaline response (Davis *et al.*, 2002), may govern the expression of a subset of these pH-dependent Rim101p-independent ORFs.

The pH response in *C. albicans* and in *S. cerevisiae*

Two recent studies analysed the transcriptional response of *S. cerevisiae* cells to alkaline conditions. Lamb *et al.* (2001) studied adaptation to pH 8, 2 h after a shift from a pH 4 grown culture. Serrano *et al.* (2002) studied adaptation to pH 7.6, 5–45 min after a shift from pH \approx 5.5. Our analyses focused on cells adapted to pH 4 or pH 8, 4 h after a shift from pH \approx 5.5. While the conditions used and the time points analysed are distinct in these three studies of fungal pH responses, there are some similarities. For example, as in *C. albicans* (Table 3), *S. cerevisiae* genes encoding ion transport proteins, iron and phosphate transporters in particular, and genes encoding stress response proteins, such as *SOD1* and *CCP1*, were induced by alkaline conditions (Lamb *et al.*, 2001; Serrano *et al.*, 2002). Furthermore, *C. albicans* homologues of *S. cerevisiae* genes involved in arginine biosynthesis (*ARG1*, *ARG3*, *ARG4*, *ARG5,6* and *CPA2*) genes involved in one-carbon metabolism (*GCV1*, *GCV2*, *SHM1* and *SHM2*) and genes involved in protein synthesis (*RPS7* and *RPL5*) were repressed at alkaline pH (Serrano *et al.*, 2002). Thus, the pH response in *C. albicans* and *S. cerevisiae* has several conserved features that may reflect general mechanisms used to adapt to environmental pH.

Using *S. cerevisiae* microarrays, Lamb and Mitchell (2003) identified 35 Rim101p-dependent genes. Several homologues of these genes were also Rim101 dependent in *C. albicans*, including *ARN1* and *YNL274* (Table 5 and data not shown). However, homologues of many *C. albicans* Rim101p-dependent ORFs are not co-ordinately regulated by Rim101p in *S. cerevisiae*. While differences in experimental conditions between these two studies may account for some of these results, it also suggests that the response of these organisms to environmental pH and the function of Rim101p in this response has changed since these two species diverged. For example, in *S. cerevisiae*, Rim101p governs sporulation and meiosis (Su and Mitchell, 1993), whereas in *C. albicans*, which has no known meiotic cycle, Rim101p promotes hyphal formation (Ramon *et al.*, 1999; Davis *et al.*, 2000a; Bennett and Johnson, 2003).

In *S. cerevisiae*, Rim101p promotes alkaline growth primarily by repressing *NRG1* transcription. The *NRG1* gene product is a Tup1p-associated protein that represses a sodium pump required for growth in alkaline and high-salt environments. In contrast, in *C. albicans*, Rim101p pro-

motes alkaline growth and hyphal formation in an Nrg1p-independent manner (Fig. 7B). However, we found that a predicted sodium efflux pump, encoded by *ENA2*, is still regulated in a pH- and Rim101p-dependent fashion. This suggests that facets of adaptation to alkaline pH is similar in *C. albicans* and *S. cerevisiae*, such as through upregulation of Na⁺-ATPases, whereas the regulation of these adaptive mechanisms may be distinct.

Brown and co-workers analysed the transcriptional profiles of 2001 *C. albicans* ORFs from wild-type and *nrg1*^{-/-} strains (Murad *et al.*, 2001b). These 2001 ORFs include 22 alkaline-up- Rim101p-dependent ORFs. Analysis of this data set reveals that pH- and Rim101p-dependent ORFs that are not associated with hyphal formation, such as *PRA1* and *PHR1*, are not differentially regulated in the *nrg1*^{-/-} mutant compared with wild-type. Rim101p-dependent genes that are associated with hyphal formation, such as *ECE1*, *HWP1*, *IHD1* and *CSA1/WAP1* (Birse *et al.*, 1993; Staab and Sundstrom, 1998; Sharkey *et al.*, 1999; Braun *et al.*, 2000; Lamarre *et al.*, 2000; Nantel *et al.*, 2002), show opposite expression patterns in the *rim101*^{-/-} and *nrg1*^{-/-} mutants. As Rim101p acts to promote hyphal formation and Nrg1p acts to repress hyphal formation in apparently parallel pathways, the differential affect on hyphal specific genes is expected. Taken together, analysis of growth and transcriptional levels provides strong support for the idea that CaRim101p does not regulate alkaline responses through repression of *NRG1*.

The pH response and iron

Extracellular pH has an important effect on iron bioavailability. In alkaline environments, iron is found in the more insoluble ferric, Fe³⁺, form and cells must respond to starvation for this essential metal. Several alkaline-up-ORFs are likely to be important for the adaptation to iron starvation. These include two iron permeases and five ferric reductases, which should reduce Fe³⁺ to the more bioavailable Fe²⁺ form, and a vacuolar iron transporter, which may transport Fe²⁺ into the cytoplasm that was taken up by endocytic vesicles as Fe³⁺ and reduced to Fe²⁺ during vesicular acidification. Finally, *ARN1*, which encodes an iron-siderophore binding protein, is an alkaline-up-ORF. Furthermore, we have found that Rim101p is required for the induction of many of these genes at alkaline pH. Clearly, iron is not the only cation that becomes limiting at high pH. For example, *ZRT1*, which encodes a high-affinity zinc transporter, is an alkaline pH- and Rim101p-dependent ORF. However, the requirement for Rim101p in regulation of iron uptake systems in alkaline environments appears to be conserved in fungi (Lamb *et al.*, 2001; Lamb and Mitchell, 2003; Eisendle *et al.*, 2004).

In acidic environments, iron is found in the soluble ferrous, Fe²⁺, form which can accumulate in cells and generate reactive oxygen species. Several alkaline-down-ORFs may be important for adaptation to excess iron and the oxidative stresses associated with it. These include genes for haem biosynthetic proteins and electron transport proteins, which contain iron either in a haem prosthetic group or in an Fe/S cluster, and may function as potential storage sites for excess intracellular iron. Three alkaline-down-ORFs encode proteins that metabolize molecules that cause oxidative damage, including catalase, cytochrome C peroxidase and Mn-superoxide dismutase, and two additional alkaline-down-ORFs encode DNA repair enzymes, *DDR48* and *RAD10*. This is consistent with the fact that the primary target of hydroxyl radical damage is DNA. Finally, aconitase, an Fe/S protein of the citric acid cycle, is an alkaline-down-ORF. In both prokaryotes and higher eukaryotes, aconitases post-transcriptionally regulate genes encoding iron responsive proteins, including ferritin and the transferrin receptor.

Environmental pH as a virulence regulator

The *RIM101* pathway is required for wild-type levels of virulence in the haematogenously disseminated murine model of candidiasis (Davis *et al.*, 2000b). Environmental pH is one potent inducer of the yeast-hyphal transition, which is a critical virulence attribute (Mitchell, 1998; Berman and Sudbery, 2002; Gow *et al.*, 2002). Our analyses of pH-responsive genes revealed that many genes important for virulence are pH regulated, including transporters important for drug resistance, iron acquisition systems and oxidative stress response proteins (Prasad *et al.*, 1995; White, 1997; Lopez-Ribot *et al.*, 1998; Hamilton and Holdom, 1999; Moye-Rowley, 2002). Rim101p, which promotes alkaline-induced hyphal formation, also governs the expression of many of these virulence genes (Ramon *et al.*, 1999; Davis *et al.*, 2000a). Thus, in *C. albicans*, environmental pH affects the expression of a broad range of cellular functions that contribute to virulence and Rim101p is required for many, but not all, of these responses.

Experimental procedures

Strains and plasmids

All *C. albicans* strains used in this study are derivatives of BWP17 and are listed in Table 8. *NRG1* was deleted in the wild-type and *rim101*^{-/-} backgrounds as follows. Strains BWP17 and DAY432 were subjected to sequential rounds of transformation as described previously (Wilson *et al.*, 1999). The *nrg1::ARG4* disruption cassette was amplified in a PCR using primers NRG1-5DR and NRG1-3DR (Table 9) and plasmid pRS-ARG4ΔSpel (Wilson *et al.*, 1999) and used to generate the *NRG1*⁺-heterozygous strains DAY587, DAY588,

DAY590 and DAY591. The *nrg1::URA3* and *nrg1::URA3-dpl220* disruption cassettes were amplified in a PCR using primers NRG1-5DR and NRG1-3DR (Table 9) and plasmids pGEM-URA3 or pDDB57 respectively (Wilson *et al.*, 1999; 2000). The *nrg1::URA3* cassette was used to generate the *nrg1*^{-/-} homozygous strains DAY593-DAY596 and DAY599-DAY601 and the *nrg1::URA3-dpl200* cassette was used to generate the *nrg1*^{-/-} homozygous strains DAY603 and DAY607. The disruption cassettes delete sequences from +5 to +924, removing residues 2–308 of the 311 amino acid Nrg1 protein sequence. Correct integration was determined by the PCR and Southern blot hybridization. PCR determination was accomplished using primers NRG1-5detect and NRG1-3detect that flank the site of integration (Fig. 1A and B). Southern blot determination (Fig. 1C and D) was accomplished by digestion of genomic DNA with *Bgl*II and separation in an 0.8% agarose gel. An *NRG1* probe was amplified via the PCR using primers NRG1-5detect and NRG1-3detect and radiolabelled with [α -³²P]-dCTP using the Prime-a-Gene labelling system (Promega). Northern blot hybridizations also showed no *NRG1* message in the *nrg1*^{-/-} and *rim101*^{-/-} *nrg1*^{-/-} strains confirming the creation of a null (Fig. 1D).

For *RIM101* complementation studies, pDDB209 (Li *et al.*, 2004) was digested with *Nru*I and transformed into DAY5 and DAY593 to generate DAY463 and DAY774 respectively. For *NRG1* complementation studies, wild-type *NRG1* coding sequence with flanking promoter and terminator sequence was amplified in a PCR using primers NRG1 5comp and NRG1 3comp from BWP17 genomic DNA. The resulting PCR product was co-transformed into a *trp1*- *S. cerevisiae* strain with *Eco*RI/*Not*I-digested pDDB78 to generate pDDB275 by *in vivo* recombination. Next, pDDB275 was digested with *Nhe*I and transformed into DAY593 and DAY599 to generate DAY808 and DAY814 respectively. DAY778 and DAY816 was generated by transforming DAY593 and DAY599 with *Nru*I-digested pGEM-HIS respectively (Wilson *et al.*, 1999).

Media and growth conditions

Candida albicans was routinely grown at 30°C in YPD (2% Bacto-peptone, 1% yeast extract, 2% dextrose). For phenotypic characterization, strains were pre-grown in YPD at 30°C, serially diluted in ddH₂O, and 3 μ l spotted onto YPD, YPD + 150 mM LiCl, YPD + 150 mM Hepes buffered at pH 9, and M199 medium (Gibco BRL) + 150 mM Hepes buffered at pH 8. All plates were grown at 37°C. For growth assays with an iron chelator, strains were pre-grown in YPD at 30°C, serially diluted in ddH₂O, and then 3 μ l spotted onto YPD + 150 mM Hepes buffered at pH 8, YPD + 150 μ M BPS + 150 mM Hepes buffered at pH 8, and YPD + 150 μ M BPS + 150 mM Hepes buffered at pH 6. For growth in iron depleted medium, strains were pre-grown in YPD at 30°C, diluted 1000 times into YPD + 0.5 mM BPS and grown for \approx 20 h. Cells were then pelleted, washed with iron-depleted medium [0.17% SD-Fe-Cu (YNB w/o Cu, Fe (Bio101)), 0.5% N₂H₄SO₄, 2% glucose, 150 mM Hepes, pH 4 or pH 8 was mixed, treated with 2.5% Chelex-100 resin (Bio-Rad) for 4 h, and filter sterilized], resuspended in iron-depleted medium at 5 \times 10⁵ cells ml⁻¹ and grown at 30°C. All media except those used for selection of Ura⁺ transformants were supplemented with 80 μ g ml⁻¹ uridine. Solid media were prepared as described above with the addition of 2% Bacto-agar.

Table 8. Strains used in this study.

Strain	Parent/ background	Relevant genotype	Reference
BWP17	SC5314	<i>ura3::λimm434/ura3::λimm434 arg4::hisG/arg4::hisG his1::hisG/his1::hisG</i>	Wilson <i>et al.</i> (1999)
DAY5	BWP17	<i>ura3::λimm434/ura3::λimm434 arg4::hisG/arg4::hisG his1::hisG/his1::hisG rim101::ARG4/ rim101::URA3</i>	Wilson <i>et al.</i> (1999)
DAY25	DAY5	<i>ura3::λimm434/ura3::λimm434 arg4::hisG/arg4::hisG HIS1::his1::hisG/his1::hisG rim101::ARG4/rim101::URA3</i>	Davis <i>et al.</i> (2000b)
DAY44	DAY5	<i>ura3::λimm434/ura3::λimm434 arg4::hisG/arg4::hisG his1::hisG/his1::hisG RIM101:: HIS1::rim101::ARG4/rim101::URA3</i>	Davis <i>et al.</i> (2000a)
DAY185	DAY286	<i>ura3Δ::λimm434/ura3Δ::λimm434 HIS1::his1::hisG/his1::hisG ARG4::URA3::arg4::hisG/ arg4::hisG</i>	Davis <i>et al.</i> (2000b)
DAY286	BWP17	<i>ura3::λimm434/ura3::λimm434 ARG4::URA3::arg4::hisG/arg4::hisG his1::hisG/his1::hisG</i>	Davis <i>et al.</i> (2002)
DAY432	BWP17	<i>ura3::λimm434/ura3::λimm434 arg4::hisG/arg4::hisG HIS1::his1::hisG/his1::hisG rim101::dpl200/rim101::dpl200</i>	Davis <i>et al.</i> (2002)
DAY463	DAY432	<i>ura3::λimm434/ura3::λimm434 arg4::hisG/arg4::hisG HIS1::RIM101::his1::hisG/his1::hisG rim101::ARG4/rim101::URA3</i>	This study
DAY587	BWP17	<i>ura3::λimm434/ura3::λimm434 arg4::hisG/arg4::hisG his1::hisG/his1::hisG nrg1::ARG4/ NRG1</i>	This study
DAY588	BWP17	<i>ura3::λimm434/ura3::λimm434 arg4::hisG/arg4::hisG his1::hisG/his1::hisG nrg1::ARG4/ NRG1</i>	This study
DAY590	BWP17	<i>ura3::λimm434/ura3::λimm434 arg4::hisG/arg4::hisG his1::hisG/his1::hisG rim101::dpl200/ rim101::dpl200 nrg1::ARG4/NRG1</i>	This study
DAY591	BWP17	<i>ura3::λimm434/ura3::λimm434 arg4::hisG/arg4::hisG his1::hisG/his1::hisG rim101::dpl200/ rim101::dpl200 nrg1::ARG4/NRG1</i>	This study
DAY593	DAY590	<i>ura3::λimm434/ura3::λimm434 arg4::hisG/arg4::hisG his1::hisG/his1::hisG rim101::dpl200/ rim101::dpl200 nrg1::ARG4/nrg1::URA3</i>	This study
DAY594	DAY590	<i>ura3::λimm434/ura3::λimm434 arg4::hisG/arg4::hisG his1::hisG/his1::hisG rim101::dpl200/ rim101::dpl200 nrg1::ARG4/nrg1::URA3</i>	This study
DAY595	DAY591	<i>ura3::λimm434/ura3::λimm434 arg4::hisG/arg4::hisG his1::hisG/his1::hisG rim101::dpl200/ rim101::dpl200 nrg1::ARG4/nrg1::URA3</i>	This study
DAY596	DAY591	<i>ura3::λimm434/ura3::λimm434 arg4::hisG/arg4::hisG his1::hisG/his1::hisG rim101::dpl200/ rim101::dpl200 nrg1::ARG4/nrg1::URA3</i>	This study
DAY599	DAY588	<i>ura3::λimm434/ura3::λimm434 arg4::hisG/arg4::hisG his1::hisG/his1::hisG nrg1::ARG4/ nrg1::URA3</i>	This study
DAY600	DAY587	<i>ura3::λimm434/ura3::λimm434 arg4::hisG/arg4::hisG his1::hisG/his1::hisG nrg1::ARG4/ nrg1::URA3</i>	This study
DAY601	DAY587	<i>ura3::λimm434/ura3::λimm434 arg4::hisG/arg4::hisG his1::hisG/his1::hisG nrg1::ARG4/ nrg1::URA3</i>	This study
DAY603	DAY588	<i>ura3::λimm434/ura3::λimm434 arg4::hisG/arg4::hisG his1::hisG/his1::hisG nrg1::ARG4/ nrg1::URA3-dpl200</i>	This study
DAY607	DAY591	<i>ura3::λimm434/ura3::λimm434 arg4::hisG/arg4::hisG his1::hisG/his1::hisG rim101::dpl200/ rim101::dpl200 nrg1::ARG4/nrg1::URA3-dpl200</i>	This study
DAY749	CT123-2	<i>ura3::λimm434/ura3::λimm434 arg4::hisG/arg4::hisG HIS1::FTR1::his1::hisG/his1::hisG ftr1::ARG4/ftr1::URA3</i>	Santos <i>et al.</i> (2003)
DAY750	CT128-1	<i>ura3::λimm434/ura3::λimm434 arg4::hisG/arg4::hisG HIS1::his1::hisG/his1::hisG ftr1::ARG4/ftr1::URA3</i>	Santos <i>et al.</i> (2003)
DAY774	DAY593	<i>ura3::λimm434/ura3::λimm434 arg4::hisG/arg4::hisG HIS1::RIM101::his1::hisG/his1::hisG rim101::dpl200/rim101::dpl200 nrg1::ARG4/nrg1::URA3-dpl200</i>	This study
DAY778	DAY593	<i>ura3::λimm434/ura3::λimm434 arg4::hisG/arg4::hisG HIS1::his1::hisG/his1::hisG rim101::dpl200/rim101::dpl200 nrg1::ARG4/nrg1::URA3-dpl200</i>	This study
DAY808	DAY593	<i>ura3::λimm434/ura3::λimm434 arg4::hisG/arg4::hisG his1::hisG/his1::hisG rim101::dpl200/ rim101::dpl200 NRG1::HIS1::nrg1::ARG4/ nrg1::URA3-dpl200</i>	This study
DAY814	DAY599	<i>ura3::λimm434/ura3::λimm434 arg4::hisG/arg4::hisG his1::hisG/his1::hisG NRG1::HIS1::nrg1::ARG4/nrg1::URA3-dpl200</i>	This study
DAY816	DAY599	<i>ura3::λimm434/ura3::λimm434 arg4::hisG/arg4::hisG HIS1::his1::hisG/his1::hisG nrg1::ARG4/nrg1::URA3-dpl200</i>	This study

RNA preps and Northern blot analysis

For microarrays and Northern blot analyses, strains were grown overnight in YPD at 30°C. The following day, cells were pelleted, washed with M199 medium at either pH 4 or pH 8 and diluted 40-fold into fresh M199 pH 4 or pH 8 medium pre-warmed to 37°C and cell density was determined by OD₆₀₀.

Cells were incubated for 4 h at 37°C with shaking. Cells were then harvested by vacuum filtration, frozen in a dry ice-EtOH bath and stored at -80°C. RNA was purified from the frozen cells as described (Adams *et al.*, 1997).

For Northern blot analyses, 15 µg of total RNA was dried, resuspended in 2× sample buffered and separated by 1.2% formaldehyde gel electrophoresis. Separated RNA was trans-

Table 9. Primers used in this study.

Name	Sequence (5'(r)3')	Reference
NRG1-5DR	TGTTTCTCATCTCAAATTTTTCCCTGCTAGTTTCATTAAGAATCAAACAATCATTATGCTttccagtcacgcggtt	This study
NRG1-3DR	CAATTAACCATCAAATTTAACCCGTTTTATAATACAATTTTGACCACATCTATACTAGGgtggaattgtgagcgata	This study
NRG1-5detect	attgttcaagtatctccag	This study
NRG1-3detect	ggcagctactatattggagg	This study
ENA2 5'detect	tgataacaataataaATGTCG	This study
ENA2 3'detect	GTGACACCCAAGATGTTACC	This study
ZRT1 5'detect	TACCCATTTATTTTTTCATCG	This study
ZRT1 3'detect	TACCAATAAGAATGGAGTGG	This study
6.2759 3'detect	TTTGTACTGATAAATGCGG	This study
6.2759 5'detect	TCAACCATGTCTAATTTGCC	This study
6.2929 5'detect	ACTTTGGGTTTGCTTTTGGG	This study
6.2929 3'detect	TGTTGTTACCAGCACCTTCG	This study
5' 6.2939	ACCTTACATGGAAAGTTGAG	This study
3' 6.2939	GGTAGCGTTATAAGTAACTG	This study
5' 6.5731	TCAGTTAATAACTTTCTTGC	This study
3' 6.5731	TGATACATGATAAAAAGTTGC	This study
5' 6.8441	AAGTACCAGTGGATTGCGTG	This study
3' 6.8441	AAGTAAACATGCAGGCCAGC	This study
5' 6.5454	aaaaaATGACAGGTTCAACC	This study
3' 6.5454	TGAGGCATAAACAGTAACGG	This study
ENB1 - 913	GTACTTGTTCGAGAAAAGTGC	This study
ENB1 + 2131	GTGTATGTGTGAATAGTGAGC	This study
RBT5 5'	CTCGCCTTATCCTTATTGTC	This study
RBT5 3'	taattcaacTTAGAATAAGG	This study
NRG1 5comp	AAGCTCGGAATTAACCCTCACTAAAGGGAACAAAAGCTGGaaataatctctgcaagagatac	This study
NRG1 3comp	ACGACGGCCAGTGAATTGTAATACGACTCACTATAGGGCGattatgacattattctgttgac	This study
PRA1 5'	ggggaattcgcaacaatatctcgttgg	Davis <i>et al.</i> (2000a)
PRA1 3'	ggggaattcgctgaactaacaattaacagtgg	Davis <i>et al.</i> (2000a)
TEF1-5'	atagtcataatcaatcATGGGT	Davis <i>et al.</i> (2000a)
TEF1-3'	cttacaataattcaactagc	Davis <i>et al.</i> (2000a)

ferred to nylon membranes by capillary action and cross-linked. Probes were made by the PCR using the primers described (Table 9) except for the *HWP1* and *ECE1* probes, which were made as described previously (Davis *et al.*, 2002). Standard PCRs contained: 1 × PCR buffer (NEB), 4 mM MgCl₂, 400 μM dNTP mix, 400 nM 5' primer, 400 nM 3' primer, ≈200 nM *C. albicans* genomic DNA, 2.5 U Taq polymerase (NEB). Typical reaction conditions were 94°C 5 min followed by 31 cycles of 94°C 45 min, 50°C 1 min, 72°C 1–2 min, followed by a 72°C 10 min step. PCR products were gel purified and radiolabelled using the Prime-a-Gene labelling system (Promega). Blots were hybridized overnight in formamide hybridization buffer at 42°C as described (Sambrook and Russell, 2001). Blots were washed with 2× SSC 0.1% SDS at 23°C for 15 min followed by one to two washes with 0.1× SSC 0.1% SDS at 50°C for 20 min. Blots were exposed to a phosphorimager screen (Molecular Dynamics) and results were quantified with IQMAC v1.2.

Microarray construction and analysis

Primer pairs were designed for 6175 unique *C. albicans* ORFs using the primer selection software Primers3 (Rozen and Skaletsky, 2000) and the *C. albicans* genome sequence (assembly 6) obtained from the Stanford Genome Technology website at <http://www-sequence.stanford.edu/group/>

candida. A complete list of primer pairs (synthesized by Lois Hoyer, University of Illinois at Urbana-Champaign, Urbana, IL) is available at <http://www.cbs.umn.edu/labs/berman/sup.pHresponse.htm>. PCR fragments were amplified from SC5314 genomic DNA, purified on MultiScreen-PCR 96-well filtration units (Millipore) and eluted in spotting buffer (3× SSC + 0.01% SDS). Purified PCR products were spotted in duplicate on poly-L-lysine-coated slides using a MicroGrid II (BioRobotics) and the slides were post-processed with succinic anhydride in 1-methyl-2-pyrrolidinone (Bensen *et al.*, 2002).

Microarray experiments were performed as described previously (Bensen *et al.*, 2002). At least four hybridizations were performed for each condition and included dye-swap experiments. In brief, 10 μg of total RNA was annealed with oligo-dT primer (Invitrogen) followed by reverse transcription in the presence of 5-(3-aminoallyl)-2'-deoxyuridine 5'-triphosphate (aa-dUTP). Samples were coupled with Cy3 or Cy5 monoreactive dye (Amersham Biosciences) and mixed. Labelled probe was resuspended in DIG Easy Hyb (Roche), heated at 65°C for 5 min, applied to the microarray and incubated at 37°C for 12–16 h. Arrays were visualized on a ScanArray 5000 microarray scanner (Packard Biosci). Scanned images were quantified with GenePix Pro 4.1 (Axon Instruments). To remove spots with low intensities and/or high background, spots that did not have at least 60% of their pixels more than one standard deviation above background

in both the Cy5 and Cy3 channels were disregarded. A visual scan of each spot was also performed to remove spots with abnormal morphologies. Median pixel intensities of spots passing our quality criteria were loaded into GeneTraffic 2.6 (Iobion Informatics) and normalized using a global lowest fit. Normalized data were analysed using SAM 1.21 one class response (Tusher *et al.*, 2001) to identify genes with statistically significant differential expression under each condition tested (Table 1). Only those genes with four or more spots passing our quality criteria were included in SAM analysis. The median number of falsely called genes by SAM was 0.49 [wild type (WT) pH 4 versus WT pH 8], 0.73 (WT pH 4 versus *rim101*^{-/-} pH 4), 0.66 (WT pH 8 versus *rim101*^{-/-} pH 8) and 0.82 (WT pH 4 versus WT pH 4). All supplemental material including array primer pairs, the raw array data and the statistically significant array data can be found at <http://www.cbs.umn.edu/labs/berman/sup.pHresponse.htm>

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