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The Transcription Factor Rim101p Governs Ion Tolerance and Cell Differentiation by Direct Repression of the Regulatory Genes *NRG1* and *SMP1* in *Saccharomyces cerevisiae*

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Environmental pH changes have broad consequences for growth and differentiation. The best-understood eukaryotic pH response pathway acts through the zinc-finger transcription factor PacC of *Aspergillus nidulans*, which activates alkaline pH-induced genes directly. We show here that *Saccharomyces cerevisiae* Rim101p, the pH response regulator homologous to PacC, functions as a repressor in vivo. Chromatin immunoprecipitation assays show that Rim101p is associated in vivo with the promoters of seven Rim101p-repressed genes. A reporter gene containing deduced Rim101p binding sites is negatively regulated by Rim101p and is associated with Rim101p in vivo. Deletion mutations of the Rim101p repression targets *NRG1* and *SMP1* suppress *rim101*Δ mutant defects in ion tolerance, haploid invasive growth, and sporulation. Therefore, transcriptional repression is the main biological function of Rim101p. The Rim101p repression target Nrg1p is in turn required for repression of two alkaline pH-inducible genes, including the Na^+ pump gene *ENA1*, which is required for ion tolerance. Thus, Nrg1p, a known transcriptional repressor, functions as an inhibitor of alkaline pH responses. Our findings stand in contrast to the well-characterized function of PacC as a direct activator of alkaline pH-induced genes yet explain many aspects of Rim101p and PacC function in other organisms.

One environmental feature with broad consequences for adaptation and differentiation is extracellular pH. In the yeast *Saccharomyces cerevisiae*, extracellular pH governs expression of genes specifying ion pumps and transporters that promote adaptation to changes in pH (4, 18, 26, 45). Extracellular pH also governs two differentiation programs, i.e., haploid invasive growth and sporulation; these are inhibited in acidic conditions and are favored in alkaline conditions (19, 28). Several of these responses depend upon a conserved regulatory pathway that acts through the transcription factor Rim101p (6, 26, 28, 38, 44). Our focus here is to determine the molecular mechanism by which Rim101p governs pH-dependent responses.

Rim101p, a C_2H_2 zinc-finger protein, was first identified through mutant analysis as a positive regulator of meiotic gene expression and sporulation (44). Epistasis analysis argued that Rim101p is part of a pathway or complex that also includes Rim8p, Rim9p, and Rim13p (44). The possibility that these gene products act in a pH response pathway came from the finding that the *Aspergillus nidulans* pH response regulator PacC is a homolog of Rim101p (46). PacC and, as subsequently found, Rim101p are activated by C-terminal proteolytic cleavage that is stimulated at alkaline pH (28, 36). Several gene products required for PacC and Rim101p cleavage are homologous to one another and include the *S. cerevisiae* calpain-like protease Rim13p (also called Cpl1p), the protease scaffold Rim20p, the putative transmembrane proteins Rim9p and Rim21p, and Rim8p, of unknown biochemical function (5, 15, 26, 52). Studies with *Yarrowia lipolytica* and *Candida albicans* have established that Rim101p and its processing pathway are conserved and that they are required for pH-dependent responses (5, 11, 16, 27, 39, 40, 47, 50). Homologs of Rim13p and Rim20p are found in metazoans, so aspects of the Rim101p processing reaction may occur in diverse eukaryotes.

Most phenotypes of *S. cerevisiae rim101* mutants are consistent with the idea that Rim101p is a positive regulator of alkaline pH-induced responses. For example, *rim101* mutants fail to undergo the alkaline pH-stimulated differentiation pathways—haploid invasive growth and sporulation (19, 28). In addition, *rim101*Δ mutants have reduced expression of several alkaline pH-induced genes (26). Finally, *rim101* mutants grow poorly in alkaline media (15, 26). However, *RIM101* has roles that may extend beyond pH-dependent response regulation. For example, *S. cerevisiae rim101* mutants are sensitive to Na^+ or Li^+ ions and grow poorly at low temperatures (26, 44). *C. albicans rim101* mutants are also sensitive to Li^+ ions (D. A. Davis et al., submitted for publication), and *Y. lipolytica rim101* mutants are defective in mating (27). These observations suggest that Rim101p has a broader role than simply to promote alkaline pH-inducible responses.

The paradigm for Rim101p functional activity comes from extensive studies of *A. nidulans* PacC (6, 13, 38). PacC is required to activate expression of alkaline pH-induced genes, such as *ipnA*, and to repress transcription of acidic pH-induced genes, such as *gabA* (13, 21, 46). PacC binds to TGCCARG-containing sequences (PacC sites) found in target promoter regions (14). Mutation of the PacC sites in the *ipnA* promoter blocks alkaline pH induction of *ipnA*, suggesting that PacC is a transcriptional activator (13). However, in the acidic pH-induced *gabA* promoter, the PacC sites overlap with sites for

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TABLE 1. Yeast strains

Strain	Background	Genotype
AMP620	SK-1[a]	*MAT***a** *trp1-hisG met4*
AMP1293	SK-1	*MAT***a** *trp1-hisG met4 tup1-269*
TLY869	SK-1	*MAT***a**
TLY870	SK-1	*MAT*α
TLY907	YC11[b]	*MAT***a** *ura3-52–URA3–CYC1*$_{PacC}$*–lacZ RIM101*::*RIM101-HA2*
TLY909	YC11	*MAT***a** *ura3-52–URA3–CYC1*$_{PacC}$*–lacZ*
TLY912	YC11	*MAT***a** *ura3-52–URA3–CYC1*$_{PacC}$*–lacZ RIM101*::*RIM101-HA2 rim13*Δ::*His3MX6*
TLY925	YC11	*MAT***a** *ura3-52–URA3 rim101* Δ*His3MX6*
TLY926	YC11	*MAT***a** *ura3-52–URA3–His3*
TLY928	SK-1	*MAT*α *smp1*Δ::*His3MX6*
TLY932	SK-1	*MAT***a** *rim101*Δ::*HIS3 smp1*Δ::*His3MX6*
TLY933	SK-1	*MAT*α *rim101*Δ::*His3 smp1*Δ::*His3MX6*
TLY936	SK-1	*MAT***a** *smp1*Δ::*His3MX6*
TLY941	SK-1	*MAT***a** *ura3*::*His3MX6*
TLY942	SK-1	*MAT*α *nrg1*Δ::*His3MX6*
TLY944	SK-1	*MAT***a** *nrg1*Δ::*His3MX6*
TLY945	SK-1	*MAT*α *rim101*Δ::*His3MX6 nrg1*Δ::*His3MX6*
TLY947	SK-1	*MAT***a** *nrg1*Δ::*His3MX6 rim101*::*His3MX6*
WXY170	SK-1	*MAT***a** *trp1-hisG met4 gal80*::*LEU2 RIM101-HA2*
WXY189	SK-1	*MAT***a** *trp1-hisG met4 gal80*::*LEU2 RIM101-HA2 rim13*Δ::*His3MX6*
WXY222	SK-1	*MAT***a** *trp1-hisG met4 gal80*::*LEU2 rim101*Δ::*His3MX6*
WXY278	SK-1	*MAT***a** *rim13*Δ::*His3MX6*
WXY281	SK-1	*MAT***a** *rim101*Δ::*His3MX6*
WXY289	SK-1	*MAT*α *rim101*Δ::*His3MX6*

[a] SK-1 strains all carry *ura3 his3*Δ*SK leu2-hisG lys2 ho-LYS2* unless noted otherwise.
[b] YC11 strains all carry *trp1*Δ*1 lys2-801 ade2-101 his3*Δ*200.*

IntA, a transcriptional activator. At alkaline pH PacC is thought to compete with IntA for binding (12). In this promoter, PacC apparently does not function as an activator. Similarly, in *Y. lipolytica*, the promoter region of the alkaline pH-induced *XPR2* gene contains PacC sites that do not provide upstream activation sequence (UAS) activity (31). Thus, PacC DNA binding properties are well understood, but the nature of PacC functional activity may be complex.

It is not know whether *S. cerevisiae* Rim101p functions as an activator or a repressor, since no direct targets have been defined. Formally, Rim101p is a positive regulator of the meiotic activator gene *IME1* and of several alkaline pH-induced genes (26, 44). However, neither *IME1* nor the *RIM101*-responsive alkaline pH-induced genes have PacC sites in their promoters, suggesting that they may be indirect targets. To elucidate the molecular and biological roles of Rim101p, we have identified and analyzed direct Rim101p target genes. Our results indicate that most Rim101p biological functions are exerted through transcriptional repression and that divergent target pathways separately control ion tolerance and cell differentiation.

MATERIALS AND METHODS

Yeast strains. Yeast strains (Table 1) were derived from SK-1 (24) or YC11 (*MAT***a**, *ura3-52 trp1*Δ*1 lys2-801 ade2-101 his3*Δ*200*), which was a gift from C. Horak and M. Snyder. The functional *RIM101-HA2* allele has been described elsewhere (28). The *rim101*Δ::*His3MX6*, *rim13*Δ::*His3MX6*, *nrg1*Δ::*His3MX6*, and *smp1*Δ::*His3MX6* disruptions were generated by replacing each entire open reading frame with *His3MX6* (29, 52). The *tup1-269* mutant (strain AMP1293) was provided by Lenore Neigeborn; the mutation was derived from a selection for increased *IME1* expression much as described earlier (34). The Tup1$^-$ phenotype segregated as a Mendelian trait was complemented by a *TUP1* plasmid and was linked to the *TUP1* locus in a genetic cross. The mutation is an A-to-T substitution at nucleotide 808 and causes a nonsense mutation (TAG) immediately after codon 269.

We use the acronym *ZPS1* (for zinc- and pH-regulated surface protein) to refer to yeast gene *YOL154W* (26, 30).

Growth conditions, β-galactosidase assays, and *lacZ* fusions. Yeast growth media (yeast-peptone-dextrose [YPD], yeast-peptone-acetate, and synthetic complete) were of standard composition (23). Growth tests on LiCl- and NaCl-containing YPD plates (pH 9) have been described elsewhere (26). For sporulation assays, log-phase yeast-peptone-acetate cultures were shifted into sporulation medium (2% potassium acetate plus 20 mg each of uracil, leucine and lysine per liter) at an optical density at 600 nm of 0.5 and sporulation was counted after 18 h. β-Galactosidase assays were carried out as described earlier (23, 26) on yeast grown exponentially for at least two doublings in either synthetic complete-Ura selective medium (see Table 3) or in YPD of the appropriate pH (see Table 5). The reporter plasmid pAED39 was constructed by inserting the sequence TCGAGTGCCAAGATGCCAAGACTCGAGTCTTGGCATCTTG GCAC into the *Xho*I site of LGΔ312S (17). The *ena1-lacZ* and *zps1-lacZ* (previously called *yol154w-lacZ*) integrating reporters have been described elsewhere (26).

Gene expression analysis. Poly(A)$^+$-selected RNA was purified on an oligo(dT) cellulose column and was used as a template for cDNA synthesis with the T7-$(dT)_{24}$ oligonucleotide as directed by Affymetrix. Biotin-labeled cRNA was generated using the Enzo-BioArray kit (Affymetrix). After fragmentation, the labeled cRNA was used to probe individual Affymetrix yeast DNA arrays, following the manufacturer's instructions. Hybridization signals for each array were normalized using all probe sets, and different arrays were compared with Microarray suite software (Affymetrix) by using statistical algorithms. We considered a twofold or greater change in expression significant and list those genes in Table 2.

Probes for Northern blot analysis were generated by the PCR using AMP108 (wild-type SK-1) genomic DNA as a template with the following oligonucleotide pairs (listed 5′ to 3′): *SMP1* (F-CTGCTAAATGGGTAGAAGAA and R-CTGGAGAGTTTGTCGAACTCG), *NRG1* (F-GATTGTTCCTCTCGACC AGC and R-AACACGGGTATACCGTCAAT), *PRB1* (F-CTGCATGCCTGC ACCCGACAGATCAGG and R-CAAACGATAGTGAAGAGGGA), *RIM8* (F-ATGGCCATGGAGGCCCCGGGTATGTCGTTACTGAGACTGTGG and R-GAGAAGCTTGGATCCTTAATAGTCATCACAAGGGG), *YDL038C* (F-CAAGTGTTGCTGGTATGTATCG and R-GACTAGATGATACTGTTTGG G), *YJR061W* (F-ATGCATGCGTAGTGGAGAGGATTACCTGA and R-CC GAAGGATAAGGGAACGTTT), and *CTS1* (F-GACGGAAGTATTTGGCT TCAT and R-AAGGCAGGGTACCTTGACGA). The *ENO1* oligonucleotides and Northern analysis methods have been described elsewhere (26).

Chromatin IP. Log-phase cultures were fixed with formaldehyde and lysed with glass beads, and extracts were prepared as described earlier (22). Extracts were sonicated so that the average DNA length was roughly 500 to 1,000 bp, and equal amounts of extract were incubated with antihemagglutinin (anti-HA) antibodies at 4°C overnight. Protein A-Sepharose beads were used to pull down the HA antibody conjugates, and then the beads were washed several times and eluted (22). DNA isolated from these samples is referred to as anti-HA immunoprecipitate. DNA that was in the starting material before the immunoprecipitation (IP) is referred to as whole-cell extract. PCRs were carried out to detect promoters using the following oligonucleotide pairs: *CYC1* (F-TCCGTGTGAG ACGACATCGT and R-AATATTTAGAGAAAAGAAG), *CYC1*$_{PacC}$ (F-GCA GGCTGGGAAGCATATTTG and R-AATATTTAGAGAAAAGAAG), *ACT1* (F-ATAAACCGTTTTGAAACCAAACTCG and R-TCTAAAAGCTGATGT AGTAGAAGATCC), *CTS1* (F-GACGGAAGTATTTGGCTTCAT and R-TG ATGTAAAGGAGTGACATTCT), *NRG1* (F-CCGCATGCCTGTGGCAGAT AAGCCTTTC and R-AGCCTGCAGCCAGACTGTAGA), *PRB1* (F-CTGCA TGCCTGCACCCGACAGATCAGG and R-TTGGTACCACTTCATCTTTG CTTGTTAG), *RIM8* (F-TAAGTTTCTTCTCTTCTATTC and R-TGTTTGGT CAATGCTACCG), *SMP1* (F-TACCTGTACCGTTCCCGATGA and R-CGG GTACCTTCTTCTACCCATTTAGCAG), *YDL038C* (F-GGCTGCAGTGTAA CCAGTTCAACCATTC and R-CCGAATTCTCTTGTACGATACATAGCC G), *YJR061W* (F-ATGCATGCGTAGTGGAGAGGATTACCTGA and R-AA GGTACCGCGCAGTGATAACATCATTGG), *YOR389W* (F-ATGCATGCA ACCACTTGAACAAGGGGAG and R-TCGGTACCTTGACGGTGGAATC

TABLE 2. *RIM101* responsive gene expression

*rim101*Δ/wt ratio[a,b]	*rim13*Δ/wt ratio[c]	*tup1*Δ/wt ratio[d]	Open reading frame or gene	Description	No. of PacC sites[e]	Rim101p binding[f]
42.2	52.0	7.7	*YPL277C*	Similar to *YOR389W*	1	Yes
36.8	27.9	4.9	*YJR061W*	Similar to mannosylphosphate transfer protein, Mnn4p	1	Yes
22.6	22.6	7.0	*YOR389W*	Similar to *YPL277C*	1	Yes
9.8	7.0	2.8	*RIM8*	Required for Rim101p processing	2	Yes
7.0	9.2	17.7	*YMR322C*	Similar to *YDR533C*	0	ND[i]
5.7	4.0	4.8	*SMP1*	Putative transcription factor, similar to *RLM1*	1	Yes
4.9	5.3	73.6	*FDH1*	Similar to formate dehydrogenases	0	ND
4.0	5.7	3.4	*YNL274C*	Similar to glycerate and formate dehydrogenases	1	ND
3.7	5.7	3.3	*ARN1*	Ferrichrome iron transporter	0	ND
3.7	3.7	1.8	*KTR5*	Putative mannosyltransferase	1	ND
3.5	3.2	11.2	*YDL038C*	Similar to mucin proteins	0	No
3.5	3.0	0.3	*YIL121W*	Similar to antibiotic resistance proteins	0	ND
3.0[g]	3.0	1.4	*CTS1*	Endochitinase	0	No
2.8	3.0	8.7	*NRG1*	Transcriptional repressor in glucose response pathway	1	Yes
2.8	2.1	2.6	*PRB1*	Vacuolar protease B	1	Yes
2.8	3.2	2.8	*YNL208W*	Similar to N starvation-induced protein	1	ND
2.3	2.1	1.6	*YPL088W*	Similar to aryl-alcohol dehydrogenases	1	ND
0.50	0.56	0.27	*UTR2*	Putative cell wall hydrolase	0	ND
0.50	0.36	0.91	*YGR035C*	Hypothetical protein	0	ND
0.50	0.77	2.4	*WSC4*	Putative integral membrane protein	0	ND
0.50	0.77	1.3	*YPL014W*	Hypothetical protein	0	ND
0.48	0.48	3.2	*FET4*	Low-affinity Fe(II) transporter	0	ND
0.43	0.43	1.0	*MFA1*	A-factor mating pheromone precursor	0	ND
0.43	0.43	1.4	*AGA2*	Adhesion subunit of a-agglutinin	0	ND
0.43	0.40	0.45	*BAR1*	Extracellular protease that inactivates α-factor	0	No
0.40	0.38	3.2	*YRO2*	Similar to *HSP30* heat shock protein, Yro1p	0	ND
0.40	0.48	0.90	*ARN4*	Siderophore iron transporter	0	No
0.29[h]	1.1	34.4	*SHC1*	Sporulation-specific homolog of *SKT5*	0	ND
0.22	0.43	4.7	*YOR049C*	Similar to *YER185W*, *RTA1*	0	ND
0.20	0.13	0.90	*COS8*	Subtelomeric protein	0	ND
0.17	1.1	1.2	*RIM101*	Zn finger transcriptional regulator	0	ND
0.15	0.23	2.0	*YDR133C*	Questionable open reading frame	0	ND
0.13	0.19	1.6	*CWP1*	Cell wall mannoprotein	0	ND
0.094	0.051	3.2	*YDL241W*	Hypothetical protein	0	ND
0.031[g]	0.036	5.2	*FLO10*	Flocculation protein	0	ND

[a] In all experiments, ratios above 2 indicate up-regulation in the mutant and ratios below 0.5 indicate up-regulation in the wild type (wt). The table is sorted in descending order of the *rim101*Δ/wild-type ratios.
[b] Ratio is calculated as the *rim101*Δ (WXY281) signal divided by the wild-type (TLY941) signal.
[c] Ratio is calculated as the *rim13*Δ (WXY278) signal divided by wild-type (TLY941) signal.
[d] Values reported in reference 20.
[e] Number of TGCCAAG sites within 600 bp upstream of start site.
[f] Indicates promoter enrichment in Rim101-HA2p chromatin IP (Fig. 2).
[g] Regulated only in SK-1 strains.
[h] Regulated only in YC11 strains (ratio compares TLY925 and TLY926 strains).
[i] ND, not determined.

TCATTATT), and *YPL277C* (F-CTGCATGCTCAAGCGTGCACCTTCAA CTT and R-ATGGTACCTTGACGATGGAATCGCATTCTC).

RESULTS

Gene expression analysis. To identify possible Rim101p target genes, we performed genomewide expression analysis. Wild-type and *rim101*Δ strains were grown logarithmically in rich YPD medium, and labeled samples were used to probe yeast DNA arrays. We carried out three independent comparisons of *rim101*Δ with wild-type strains in two different strain backgrounds. We found 17 genes that were up-regulated twofold or more in *rim101*Δ mutant strains and 18 genes that were down-regulated twofold or more in *rim101*Δ mutant strains (Table 2). Several of these genes have known or predicted functions in the cell wall (*YJR061W*, *KTR5*, *YDL038C*, *CTS1*, *UTR2*, *AGA2*, *SHC1*, *CWP1*, and *FLO10*), some have a role in iron uptake (*ARN1*, *FET4*, and *ARN4*), some are potential membrane proteins (*YPL277C*, *YOR389W*, *YIL121W*, and *WSC4*), and two are transcriptional regulators (*SMP1* and *NRG1*). Notably, three genes involved in the mating response—*AGA2*, *BAR1*, and *MFA1*—were down-regulated in *rim101*Δ strains; down-regulation of the homologous genes may cause the mating defect of *Y. lipolytica rim101*Δ mutants.

We used Northern analysis to confirm the array results for several genes. We focused on genes that were up-regulated in *rim101*Δ strains, as explained below. Transcripts of *CTS1*, *NRG1*, *PRB1*, *RIM8*, *SMP1*, *YDL038C*, and *YJR061W* were detected at higher levels in a *rim101*Δ strain than in an isogenic *RIM101* strain (Fig. 1, lane 2 compared to lane 1). Levels of a control *ENO1* transcript were similar in the two strains (Fig. 1). Thus, these genes are negatively regulated by Rim101p.

Based on the amino acid similarity within the zinc-finger region of Rim101p and PacC, Rim101p is predicted to bind to a PacC site (TGCCARG). Promoter region TGCCAAG sites

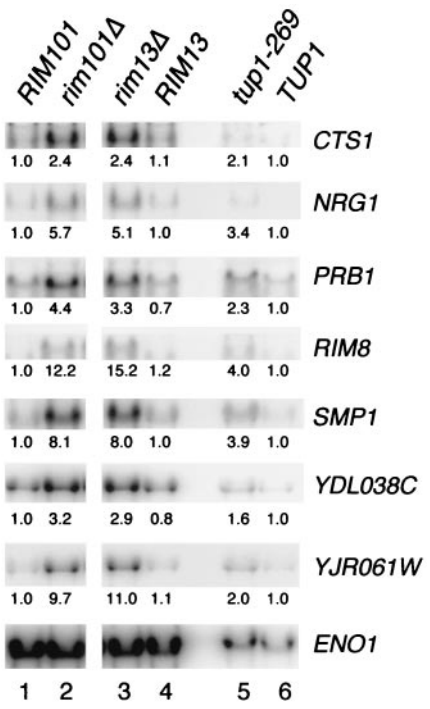

FIG. 1. Northern blot analysis of Rim101p-repressed genes. RNA prepared from YPD cultures of strains TLY941 (wild type, lanes 1 and 4), WXY281 (*rim101*Δ, lane 2), WXY278 (*rim13*Δ, lane 3), AMP1293 (*tup1-269*, lane 5), and AMP620 (wild type, lane 6) was used to prepare Northern blots, which were probed for *SMP1*, *NRG1*, *PRB1*, *RIM8*, *YDL038C*, *YJR061*, *CTS1*, and *ENO1* transcripts. Blots were visualized and quantitated with a phosphorimager. The number under each lane represents the probe signal, corrected for *ENO1* expression and setting the wild-type signal (lanes 1 or 6) at 1.0. Lanes 1 to 4 show 10 μg of poly(A)$^+$ RNA; lanes 5 and 6 show 20 μg of total RNA.

occur in most of the genes that were up-regulated in *rim101*Δ strains but not in genes that were down-regulated (Table 2). Also, analysis of the complete expression data set with the algorithm for regulatory element detection using correlation with expression (3) revealed that the presence of the 7-nucleotide motif TGCCAAG in a promoter most strongly correlated ($\Delta\chi^2 = 0.005064$) with increased expression of the downstream gene in the *rim101*Δ mutant (data not shown). Thus, if Rim101p regulates transcription directly through PacC sites in *S. cerevisiae*, then Rim101p is predicted to function as a repressor.

The role of PacC sites in *S. cerevisiae*. We used artificial reporter constructs to determine whether Rim101p acts through PacC sites and whether it functions as a repressor. Four PacC sites were inserted between the UAS and TATA region of a *CYC1-lacZ* fusion to create a reporter designated *CYC1*$_{PacC}$*-lacZ*. The *CYC1-lacZ* construct lacking PacC sites was expressed at similar high levels in both *RIM101* and *rim101*Δ strains (Table 3). *CYC1*$_{PacC}$*-lacZ* expression was 211-fold lower than that of *CYC1-lacZ* in the *RIM101* strain. Repression was almost entirely dependent on *RIM101* because *CYC1*$_{PacC}$*-lacZ* expression was only twofold lower than that of *CYC1-lacZ* in the *rim101*Δ strain (Table 3). In similar experiments, we found that placement of PacC sites in front of a promoter lacking other activation sequences did not stimulate

TABLE 3. The effect of PacC sites on transcription in the wild type and in *rim101*Δ, *rim13*Δ, and *tup1* mutants

Strain	Relevant genotype	β-Galactosidase activity[a] for:		Repression (*n*-fold)[b]
		CYC1-lacZ	*CYC1*$_{PacC}$*-lacZ*	
TLY941	*RIM101 RIM13*	1,542	7.3	211
WXY281	*rim101*Δ *RIM13*	1,759	829	2.1
WXY278	*RIM101 rim13*Δ	1,020	322	3.2
AMP620	*TUP1*	990	2.3	430
AMP1293	*tup1-269*	940	322	2.9

[a] Values are the mean of three or four determinations, and standard deviations were < 25% of the mean.
[b] Values were calculated by dividing the β-galactosidase activity of *CYC1-lacZ* by that of *CYC1*$_{PacC}$*-lacZ*.

lacZ reporter expression, regardless of the *RIM101* allele (data not shown). Thus, in this artificial context, PacC sites do not have UAS activity; instead they direct Rim101p-dependent repression. These results are consistent with the model that Rim101p functions as a repressor.

Association of Rim101p with target promoters. To determine whether Rim101p associates with target promoter regions in vivo, we carried out chromatin IP experiments (Fig. 2). We examined strains expressing wild-type Rim101p or a functional HA epitope-tagged derivative (Rim101-HAp), expressed from the *RIM101* promoter. DNA isolated from anti-HA chromatin IPs was used in PCR assays to detect target promoters (Fig. 2, lanes 1 to 4). As a control, the whole-cell extracts were used in parallel PCR assays to ensure the equivalence of the IP starting material (Fig. 2, lanes 7 to 10). We observed that the Rim101p-repressed *NRG1*, *PRB1*, *RIM8*, *SMP1*, *YJR061W*, *YOR389W*, and *YPL277C* promoter regions were enriched in the anti-HA IPs of the Rim101-HAp strain (Fig. 2, lanes 3 and 4) compared to the untagged Rim101p strain (Fig. 2, lanes 1 and 2). As an internal positive control, the *CYC1*$_{PacC}$*-lacZ* reporter had been integrated in the genome of each strain, and we observed that the *CYC1*$_{PacC}$*-lacZ* promoter was also enriched in anti-HA IPs of the Rim101-HAp strain. In contrast, promoter sequences for two other Rim101p-repressed genes (*CTS1* and *YDL038C*), two Rim101p-activated genes (*ARN4* and *BAR1*), and a Rim101p-nonresponsive gene (*ACT1*) were present at similar levels in IPs of both strains (Fig. 2). Also, the native *CYC1* promoter lacking PacC sites was present at similar levels (Fig. 2). Thus, Rim101p may act indirectly to repress *CTS1* and *YDL038C* and to activate *ARN4* and *BAR1*. However, our results indicate that Rim101p acts directly at the promoters of *NRG1*, *PRB1*, *RIM8*, *SMP1*, *YJR061W*, *YOR389W*, and *YPL277C* to cause repression.

Effect of Rim101p processing on repression and promoter association. The activity of Rim101p depends on processing by the calpain-like protease Rim13p (15, 26). In keeping with this model, we observed that *rim13*Δ and *rim101*Δ mutations caused similar gene expression alterations (Table 2 and Fig. 1). Also, repression by Rim101p through PacC sites is dependent upon Rim13p function (Table 3). These data confirm that the main function of Rim13p under these growth conditions is to promote Rim101p activity. We considered the possibility that processing by Rim13p is required for Rim101p to bind DNA in vivo. If this were the case, then association of Rim101p with

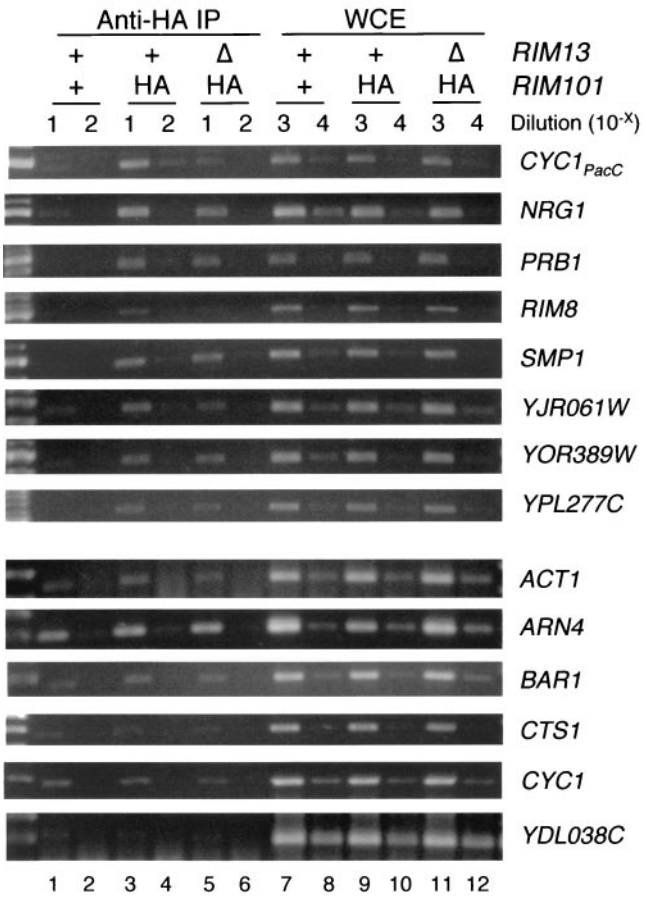

FIG. 2. Chromatin IPs to detect Rim101-HAp DNA binding in vivo. DNA from wild-type (TLY 909, lanes 1, 2, 7, and 8), *RIM101-HA* (TLY907, lanes 3, 4, 9, and 10), and *rim13*Δ *RIM101-HA* (TLY912, lanes 5, 6, 11, and 12) strains was purified from equal amounts of extract before (WCE, lanes 7 to 12) and after anti-HA chromatin IP (anti-HA IP, lanes 1 to 6). Purified DNA was diluted as indicated, and 1 μl was used as a template to detect several promoter regions in separate 50-μl PCRs. One-fifth of each reaction was separated on 1.2 to 2.0% agarose Tris-borate-EDTA gels and visualized with ethidium bromide. The *NRG1*, *RIM8*, and *BAR1* promoters were detected with 30 cycles of amplification; the $CYC1_{PacC}$, *CYC1*, *ACT1*, *CTS1*, and *YDL038C* promoters were detected with 35 cycles, and the other promoters were detected with 28 cycles.

target promoters would depend upon *RIM13*. This seems to be true for the $CYC1_{PacC}$-*lacZ* and *RIM8* promoter regions: anti-HA IP enrichment of these regions was lost in the *rim13*Δ strain (Fig. 2, lanes 4 to 6). However, most of the natural Rim101p targets, including the *NRG1*, *PRB1*, *SMP1*, *YJR061W*, *YOR389W*, and *YPL277C* promoters, were similarly enriched in anti-HA IPs from the *RIM13* and *rim13*Δ strains. Therefore, unprocessed Rim101p associates with many of these promoters in vivo, but repression is still dependent on Rim101p processing.

Requirement for Tup1p in Rim101p-dependent repression. Many Rim101p-repressed genes are also negatively regulated by the corepressor subunits Tup1p and Ssn6p (summarized for Tup1p in Table 1), based upon genomewide expression surveys (7, 20). Northern analysis confirmed that several of these genes are expressed at elevated levels in a *tup1* mutant (Fig. 1A, lanes 5 and 6). If repression by Rim101p depends upon Tup1p, then repression through PacC sites should be relieved in a *tup1* mutant. A comparison of *CYC1-lacZ* and $CYC1_{PacC}$-*lacZ* expression indicated that PacC sites direct only 2.9-fold repression in a *tup1* mutant, compared to 430-fold repression in an isogenic wild-type strain (Table 3). These results indicate that repression through PacC sites depends upon Tup1p.

The role of *NRG1* in Rim101p-dependent biological activity. The direct Rim101p target *NRG1* specifies a transcription factor. Nrg1p represses transcription of several glucose-repressed genes and, together with its close homolog Nrg2p, negatively regulates invasive growth (25, 37, 49, 53). Thus, it seemed possible that some *rim101*Δ mutant phenotypes might be due to increased expression of *NRG1*. If this hypothesis were true, then an *nrg1*Δ mutation would suppress some *rim101*Δ mutant phenotypes. The *nrg1*Δ mutation had no effect on the *rim101*Δ defects in invasive growth and sporulation (Fig. 3C and Table 4). However, the *nrg1*Δ mutation fully suppressed the *rim101*Δ defect in growth at pH 9 (Fig. 3A) and at 17°C (data not shown). In addition we observed that the *nrg1*Δ mutation confers resistance to Na^+ and Li^+ ions (Fig. 3A, compare the wild type and *nrg1*Δ) and found that Na^+ and Li^+ resistance cosegregated with *nrg1*Δ through meiosis (data not shown). In an *nrg1*Δ background, the *rim101*Δ mutation had no effect on Na^+ and Li^+ sensitivity. Therefore, increased expression of *NRG1* can account for the *rim101*Δ mutant sensitivity to alkaline pH, low temperature, and Na^+ and Li^+ ions. In addition, our results reveal a new role for Nrg1p as a negative regulator of Na^+ and Li^+ tolerance.

One way that *S. cerevisiae* adapts to alkaline pH and excess Na^+ and Li^+ is by increased expression of the Na^+ pump gene, *ENA1* (18, 45). Expression of *ENA1* partially depends on Rim101p (26). If Nrg1p acts downstream of Rim101p to govern alkaline pH, Na^+, and Li^+ sensitivity, then Nrg1p may function as a negative regulator of *ENA1*. We examined the pH response of *ena1-lacZ* to test this model (Table 5). At pH 4, the wild-type strain expressed *ena1-lacZ* at low uninduced levels, the *rim101*Δ strain expressed *ena1-lacZ* at 30-fold-lower levels, and the *nrg1*Δ mutant expressed *ena1-lacZ* at 10-fold-higher levels than did the wild-type strain. The *rim101*Δ *nrg1*Δ double mutant, like the *nrg1*Δ mutant, expressed *ena1-lacZ* at high levels. At pH 8, the wild-type strain expressed *ena1-lacZ* at induced levels, the *rim101*Δ mutant expressed *ena1-lacZ* at fourfold-lower levels, and the *nrg1*Δ and *rim101*Δ *nrg1*Δ strains expressed *ena1-lacZ* at the same high level as the wild type. Therefore, an *nrg1*Δ mutation is sufficient to increase *ena1-lacZ* expression at acidic pH and can suppress the *rim101*Δ mutant defect in alkaline pH-induced *ena1-lacZ* expression. These results support the idea that Nrg1p acts downstream of Rim101p to repress *ENA1*.

To determine whether Nrg1p governs expression of additional alkaline pH-induced genes, we also examined expression of a *zps1*(*yol154w*)-*lacZ* fusion. We verified that full levels of *zps1-lacZ* expression depend upon Rim101p (Table 5), as shown previously (26). Presence of an *nrg1*Δ mutation caused overexpression of *zps1-lacZ* at both pH 4 and pH 8 and rendered expression independent of Rim101p. Together, these results indicate that Rim101p promotes alkaline pH induction of *ENA1* and *ZPS1* by repressing the *NRG1* repressor gene.

The role of *SMP1* in Rim101p-dependent biological activity.

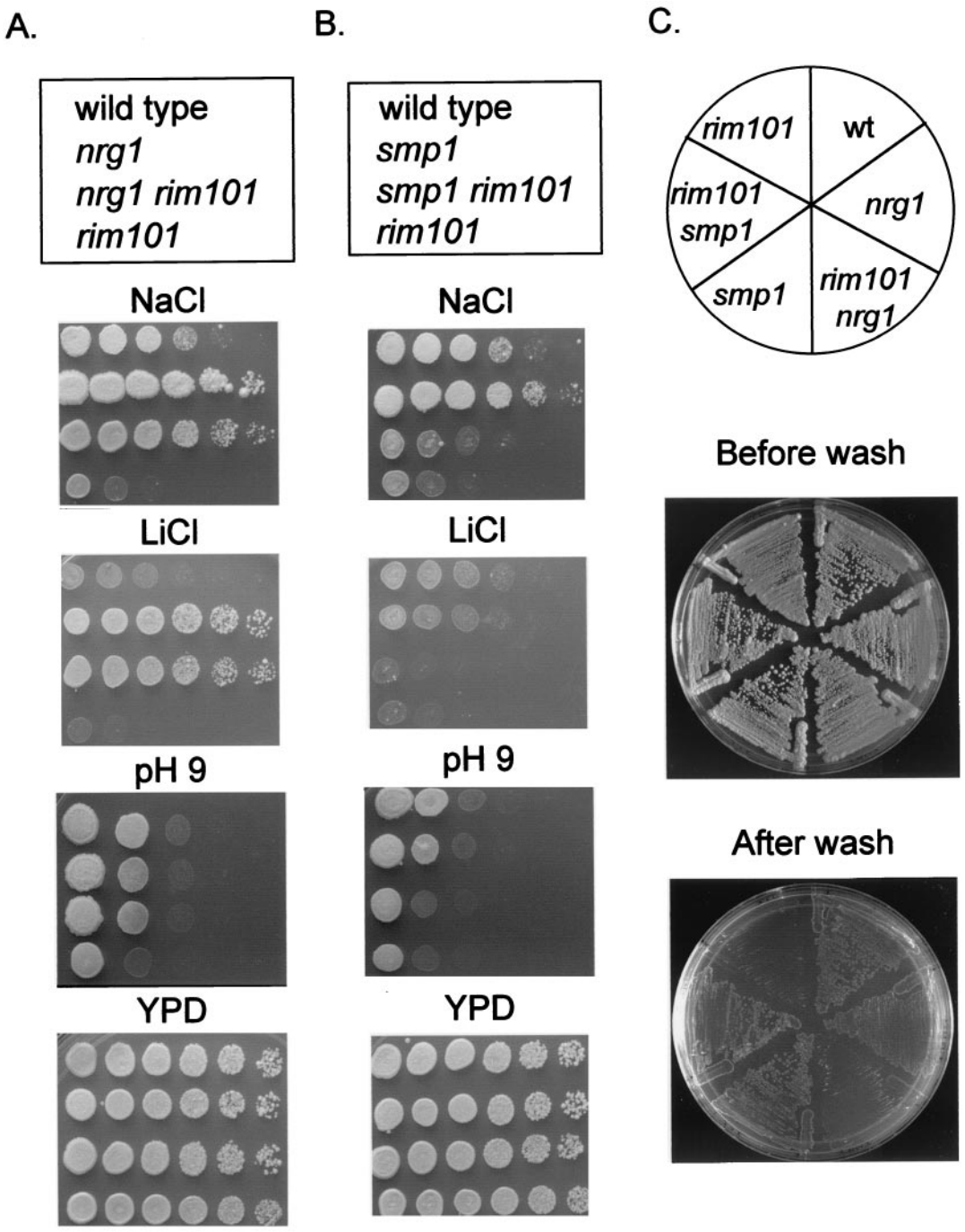

FIG. 3. Roles of *SMP1* and *NRG1* in Rim101p-dependent responses. (A) Fivefold serial dilutions of strains TLY941(*RIM101 NRG1*), WXY 281 (*rim101*Δ), TLY944 (*nrg1*Δ), and TLY947 (*rim101*Δ *nrg1*Δ) were spotted on a control YPD plate and on YPD with the following modifications: titrated to pH 9, containing 25 mM LiCl, or containing 0.4 M NaCl. (B) Fivefold serial dilutions of strains TLY941(*RIM101 SMP1*), WXY 281 (*rim101*Δ), TLY936 (*smp1*Δ), and TLY932 (*rim101*Δ *smp1*Δ) were spotted on plates as described above. (C) Invasive growth was determined by washing a YPD plate after 7 days of growth. wt, wild type.

The direct Rim101p target gene, *SMP1*, also specifies a transcription factor. Smp1p (for second MEF2-like protein) is homologous to Rlm1p, a MADS box family transcription factor that activates transcription in response to the cell integrity-Mpk1p mitogen-activated protein kinase pathway (10). However, the function of Smp1p is not known. To determine whether some *rim101*Δ defects are the result of elevated Smp1p levels, we examined whether an *smp1*Δ mutation could suppress any *rim101*Δ mutant phenotypes. The *rim101*Δ mutant defects in alkaline pH and ion tolerance were largely unaffected by the *smp1*Δ mutation (Fig. 3B). We noted that the *smp1*Δ mutation conferred Na^+ resistance but that a *rim101*Δ

TABLE 4. The roles of *RIM101*, *NRG1*, and *SMP1* in sporulation

Diploid strain	Relevant genotype			% Sporulation[a]
	RIM101	*NRG1*	*SMP1*	
TLY869 × TLY870	+/+	+/+	+/+	92 ± 2
WXY281 × WXY289	Δ/Δ	+/+	+/+	9 ± 3
TLY945 × TLY946	Δ/Δ	Δ/Δ	+/+	6 ± 3
TLY942 × TLY944	+/+	Δ/Δ	+/+	94 ± 4
TLY932 × TLY933	Δ/Δ	+/+	Δ/Δ	33 ± 3
TLY928 × TLY936	+/+	+/+	Δ/Δ	96 ± 2

[a] Percent sporulation is the average value plus or minus standard deviation for four independent diploids.

mutation caused Na^+ sensitivity in both *SMP1* and *smp1*Δ backgrounds (Fig. 3B). In keeping with these epistasis tests, the *smp1*Δ mutation had no effect on *ENA1* expression at pH 4 or pH 8 (Table 5), thus suggesting that Smp1p and Rim101p govern Na^+ tolerance through independent pathways. The *smp1*Δ mutation also had no effect on *zps1-lacZ* expression (Table 5). In contrast, the *smp1*Δ mutation fully suppressed the *rim101*Δ mutant defect in invasive growth (Fig. 3C) and partially suppressed the defect in sporulation (Table 4). The *smp1*Δ mutation also restored rough colony morphology to the otherwise smooth *rim101*Δ mutant (data not shown). These observations argue that elevated *SMP1* expression in *rim101*Δ mutants inhibits invasive growth and sporulation and promotes smooth colony morphology.

DISCUSSION

Rim101p homologs are broadly distributed among fungi, where they are required for alkaline pH-induced gene expression and diverse differentiation pathways (6, 38). Here we show that *S. cerevisiae* Rim101p exerts its biological functions primarily as a repressor, based on three lines of evidence. First, Rim101p is associated in vivo with the promoters of several genes, and these genes are negatively regulated by Rim101p. Second, an artificial reporter gene containing deduced Rim101p binding sites is negatively regulated by Rim101p. Third, deletion mutations of two Rim101p repression targets, *NRG1* and *SMP1*, each suppress a subset of *rim101*Δ mutant phenotypes. Our results show that Rim101p is associated with many target promoter regions regardless of whether it is processed or unprocessed; however, Rim101p processing is required for its activity as a repressor. As the corepressor complex Tup1p-Ssn6p also negatively regulates all direct Rim101p repression targets, it is possible that processing of Rim101p is required for functional Rim101p-Tup1p-Ssn6p interaction. Although our findings differ in several respects from the PacC paradigm, we argue that the biological and molecular repression functions of Rim101p and PacC may be conserved.

TABLE 5. The roles of *RIM101*, *NRG1*, and *SMP1* in pH-responsive gene expression

Strain	Relevant genotype for:			β-Galactosidase activity[a] for:			
				ena1-lacZ at:		*zps1-lacZ* at:	
	RIM101	*NRG1*	*SMP1*	pH 4	pH 8	pH 4	pH 8
TLY869	+	+	+	0.60	134	5.2	33
WXY281	Δ	+	+	0.02	32	1.1	9.4
TLY944	+	Δ	+	5.80	163	19.9	167
TLY947	Δ	Δ	+	2.58	125	19.3	376
TLY936	+	+	Δ	0.53	121	3.7	46
TLY932	Δ	+	Δ	0.03	36	2.6	11

[a] Values are the mean of three or four determinations, and standard deviations were < 30% of the mean for *ena1-lacZ* and were < 22% of the mean for *zps1-lacZ*.

Rim101p DNA binding, processing, and repression. We found that a functional epitope-tagged Rim101p associates with Rim101p-repressed promoter regions in vivo. Two observations indicate that Rim101p acts through the sequence TGC CAAG, a PacC site. First, the sequence appears in all Rim101p-associated promoter regions but in fewer than 10% of all *S. cerevisiae* promoters. Second, introduction of four copies of this sequence into the *CYC1* promoter confers Rim101p-dependent repression. Our results are consistent with the idea that Rim101p binds directly to the sequence TGCCAAG because Rim101p is associated with the $CYC1_{PacC}$-*lacZ* promoter but not with the *CYC1* promoter. However, repression by Rim101p through a PacC site depends upon promoter context (W. Xu and A. P. Mitchell, unpublished results). Thus, a single PacC site may be necessary but not sufficient to direct Rim101p repression in vivo.

Rim101p-DNA association is processing independent at some promoters and processing dependent at others. DNA association by unprocessed Rim101p was unexpected since unprocessed *A. nidulans* PacC is largely cytoplasmic, whereas processed PacC is exclusively nuclear (33). Processing may influence Rim101p localization in *S. cerevisiae* as well, which would explain the processing-dependent association with the *RIM8* promoter. The ability of unprocessed Rim101p to bind other target promoters could be due to the presence of higher-affinity sites. Several transcription factors are bound to target promoters in their inactive states (reviewed in reference 51). However, since repression of Rim101p target genes still depends on processing, the Rim101p C-terminal region must inhibit repression activity and cannot solely govern Rim101p DNA binding activity or intracellular localization. If Rim101p exerts repression through direct recruitment of Tup1p-Ssn6p, then a simple model is that the Rim101p C-terminal region blocks this recruitment.

Implications for the function of Rim101p/PacC homologs. Rim101p/PacC homologs have been studied primarily as direct activators of alkaline pH-induced genes (6, 38). Our findings here differ from this paradigm in that *S. cerevisiae* Rim101p functions primarily as a repressor and that it promotes alkaline pH-induced genes indirectly through repression of Nrg1p. Perhaps the biochemical function of *S. cerevisiae* Rim101p has diverged substantially from its homologs, but several observations from other fungi are consistent with our findings. First, *A. nidulans* PacC functions as a repressor at the *gabA* promoter (12). Second, *C. albicans* Rim101p is formally a negative regulator of *RIM8*/*PRR1* expression (40), as expected if direct repression of *RIM8* by Rim101p is conserved. Third, *C. albicans* Rim101p is a positive regulator, while Nrg1p is a negative regulator, of hypha-specific genes and morphogenesis (2, 5, 11, 35, 40), as expected if Rim101p repression of Nrg1p is conserved. Thus, these previous findings can be understood if the repression function of Rim101p is conserved.

Although we argue that Rim101p/PacC homologs function

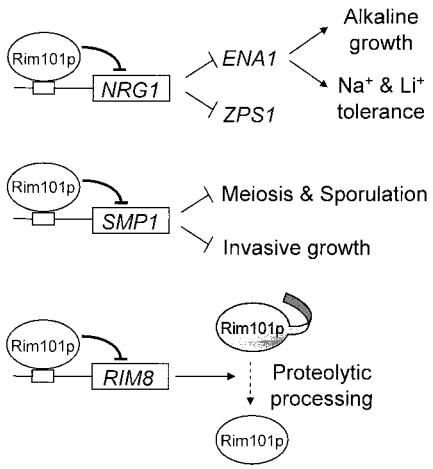

FIG. 4. Relationship of Rim101p to repression targets and biological function. Rim101p associates with promoter regions of *NRG1*, *SMP1*, *RIM8*, and other genes to cause repression. Repression and, in some cases, promoter association depend upon processing of Rim101p by Rim13p. The repression target Nrg1p functions as a negative regulator of alkaline pH-induced genes *ZPS1* and *ENA1*; Ena1p is required for alkaline growth and Na^+ and Li^+ tolerance. The repression target Smp1p functions as a negative regulator of invasive growth and sporulation, though other Rim101p targets may govern sporulation as well. The repression target Rim8p promotes Rim101p processing, and repression of *RIM8* may prevent hyperactivity of Rim101p or of the protease Rim13p.

as repressors, there is clear and compelling evidence that many also function as activators (reviewed in references 6 and 38). How might they function in both ways? One possibility is that Rim101p/PacC proteins function as repressors unless they associate with an activator. Indeed, in *Y. lipolytica*, PacC sites and an Abf1p activator site are required to create a pH-responsive UAS (31). A second possibility is that different forms of Rim101p/PacC homologs have opposite activities. PacC cleavage occurs in two steps to yield N-terminal fragments of ~500 and ~250 residues (9). The ~500-residue form—the major form in *S. cerevisiae*—may be a repressor in all organisms, while the ~250-residue form may be an activator.

The function of Rim101p target genes. The Rim101p repression target *RIM8* is required for Rim101p processing, a relationship that has properties of a negative feedback loop (Fig. 4). In *C. albicans*, a *rim101*Δ mutation also causes overexpression of *RIM8*/*PRR1* (40), so this homeostatic circuit is conserved. Repression of *RIM8* is functionally significant, because strains lacking functional Rim101p have elevated processing rates (Xu and Mitchell, unpublished). This mechanism may prevent either hyperaccumulation of processed Rim101p or hyperactivity of the Rim13p protease.

Our functional analysis here focused on two Rim101p repression targets, *NRG1* and *SMP1*, because they specify transcription factors and thus seemed likely to mediate Rim101p-dependent functions. We have identified new functions for both of these gene products. Smp1p has properties of a negative regulator of haploid invasive growth, rough colony morphology, and sporulation (Fig. 4). Smp1p is a MADS box protein homologous to Rlm1p, a target of the protein kinase C/cell integrity mitogen-activated protein kinase pathway (10). A prospective Smp1p binding site (10) occurs upstream of *CWP1*, a mannoprotein gene that promotes cell wall integrity (8, 43, 48). *CWP1* is down-regulated in the *rim101*Δ mutant, in which *SMP1* expression is elevated, as expected if Smp1p were a transcriptional repressor. This hypothesis may permit identification of direct Smp1p targets that mediate Rim101p-dependent differentiation responses.

Nrg1p has a major role in pH-responsive gene regulation and ion tolerance (Fig. 4). One key role of Nrg1p is to negatively regulate *ENA1*, an Na^+ efflux pump gene that is critical for growth in alkaline media and for Na^+ and Li^+ tolerance (18, 42, 45). Nrg1p is a repressor (37), and two possible Nrg1p binding sites (CCCCT and CCCTC) occur in the *ENA1* 5′ region at −650 and −725 in the *ENA1* 5′ region, so Nrg1p may repress *ENA1* directly. Prior studies indicate that Nrg1p activity is inhibited by the protein kinase Snf1p, which mediates glucose repression (25, 49). Snf1p is known to promote *ENA1* expression in part through inhibition of the repressor Mig1p (1), but it is possible that Snf1p also promotes *ENA1* expression through inhibition of Nrg1p. Thus, Nrg1p may couple *ENA1* expression and ion tolerance to both carbon and pH signaling pathways.

Nrg1p is a negative regulator of a second alkaline pH-induced gene, *ZPS1*. Zps1p function is uncertain, but both *ZPS1* and its *C. albicans* homolog *PRA1* are Rim101p-dependent alkaline pH-induced genes (5, 26, 41). *ZPS1* has a prospective Nrg1p binding site within its promoter (at position −190), so it may be a direct target of Nrg1p repression. Therefore, *S. cerevisiae* Rim101p activates at least two alkaline pH-induced genes through a repression relay: Rim101p represses *NRG1*, and Nrg1p in turn negatively regulates alkaline pH-induced genes.

Because Nrg1p governs pH-responsive gene expression, it is possible that Nrg2p does so as well. Nrg1p and Nrg2p are close homologs that function together to repress *FLO11*, *DOG2*, pseudohyphal growth, and biofilm formation (25, 49). For *FLO11* expression in particular, their roles seem redundant (25, 49). For several other genes, Nrg1p alone has a detectable role, though the role of Nrg2p in repression of many targets has not been examined (37, 49, 53). Our expectation is that Nrg1p has a more central role than Nrg2p during growth in acidic conditions, because *NRG1* is up-regulated at acidic pH, while *NRG2* is up-regulated at alkaline pH (4, 25). Thus, Nrg1p may function to repress alkaline pH-induced genes primarily in acidic growth conditions.

Our findings support the idea that *S. cerevisiae* pH-responsive gene expression involves the interplay of several regulatory pathways. While Rim101p and Nrg1p are important for adaptation to alkaline pH, *ENA1* and *ZPS1* are still induced at pH 8 in *rim101*Δ *nrg1*Δ double mutants. Induction of *ENA1* by alkaline pH has been shown to depend on the calcineurin-activated transcription factor Crz1p (32). Similarly, we have observed that induction of *ZPS1* by alkaline pH depends upon the zinc-responsive transcription factor Zap1p (T. M. Lamb and Mitchell, unpublished observations). Thus, Rim101p and Nrg1p control activity of the *ENA1* and *ZPS1* promoters in conjunction with other pH-responsive regulatory pathways. This interplay fits well with the finding that external pH changes have wide-ranging physiological impact, as reflected by the diverse groups of pH-responsive genes (4, 26).

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