Functional characterization of the regulators of calcineurin in *Candida glabrata*

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ABSTRACT

The serine-threonine-specific protein phosphatase calcineurin is a key mediator of various stress responses in fungi. Here, we characterized functions of the endogenous regulators of calcineurin (RCNs), Rcn1 and Rcn2, in the pathogenic fungus *Candida glabrata*. Rcn1 exerted both inhibitory and stimulatory effects on calcineurin signaling, but Rcn2 displayed only inhibitory activity. Phenotypic analyses of *C. glabrata* strains lacking either RCNs, calcineurin, or both revealed that calcineurin requires Rcn1, but not Rcn2, for antifungal tolerance in *C. glabrata*.

INTRODUCTION

The serine-threonine-specific protein phosphatase calcineurin plays critical roles in antifungal resistance and virulence in pathogenic fungi, including *Candida albicans*, *Candida glabrata*, *Cryptococcus neoformans*, and *Aspergillus fumigatus*, and has therefore attracted attention as a novel target for antifungal therapy (Steinbach*, et al.*, 2007, Miyazaki*, et al.*, 2010a). However, immunosuppressive effects of the current calcineurin inhibitors, such as FK506 and cyclosporin A, preclude the use of such inhibitors for the treatment of opportunistic fungal infections in the clinical setting. Several characterized endogenous regulators of calcineurin (RCNs) have been shown to physiologically modulate the activity of calcineurin signaling (Hilioti & Cunningham, 2003). For example, Rcn1, which was the first identified conserved member of RCNs, is presumed to exist in all fungi (Kingsbury & Cunningham, 2000, Stie & Fox, 2008); however, to date, functional homologs of Rcn1 have been characterized in only three fungal species: *Saccharomyces cerevisiae*, *C. neoformans*, and *C. albicans* (Gorlach*, et al.*, 2000, Kingsbury & Cunningham, 2000, Reedy*, et al.*, 2009). Although Rcn2 has recently been identified as another member of RCNs in *S. cerevisiae* (Mehta*, et al.*, 2009), it has yet to be studied in other fungi.

C. glabrata has emerged as an important fungal pathogen due in part to its resistance to azole antifungals such as fluconazole (Pfaller & Diekema, 2007). Recently, we demonstrated in *C. glabrata* that calcineurin is involved in responses to various types of stress through downstream transcription factor Crz1-dependent and -independent pathways (Miyazaki*, et al.*, 2010a). However, little is known about the regulation mechanisms of calcineurin in this fungus. In this study, our objective was to characterize the functions of Rcn1 and Rcn2 orthologs in *C. glabrata*.

MATERIALS AND METHODS

Strains and culture conditions

The *C. glabrata* and *S. cerevisiae* strains used in this study are listed in Table 1. Strains were routinely propagated at 30 °C in YPD medium (1% yeast extract, 2% peptone, and 2% dextrose) (Difco Laboratories, Detroit, MI), synthetic complete (SC) medium, or SC medium lacking tryptophan (Sc-trp) (Kaiser, *et al.*, 1994).

Plasmid and strain construction

Sequence information of *C. glabrata* genes was obtained from the *C. glabrata* genome database Genolevures (http://www.genolevures.org/). The primers and plasmids used in this study are listed in Tables 2 and 3, respectively. The *S. cerevisiae* and *C. glabrata RCN* genes were amplified from the genomic DNA of BY4742 (Winzeler, *et al.*, 1999) and CBS138 (Dujon, *et al.*, 2004), respectively. All plasmids constructed using PCR products were verified by sequencing before use. Transformation of *S. cerevisiae* and *C. glabrata* was performed using a lithium acetate protocol, as described previously (Cormack & Falkow, 1999).

C. glabrata deletion strains were generated using a one-step PCR–based technique, as described previously (Miyazaki, *et al.*, 2010b). Briefly, a deletion construct was amplified from either pBSK-TRP or pBSK-HIS using primers tagged with 100-bp sequences homologous to the flanking regions of the target ORF. *C. glabrata* parent strains were subsequently transformed with the deletion construct, and the resulting transformants were selected by tryptophan or histidine prototrophy, as appropriate. Both PCR and Southern blotting were performed to verify that the desired homologous recombination occurred at the

target locus without ectopic integration of the deletion construct. To generate complemented strains, the deletion mutants TG231 (Δ*rcn1*, Δ*trp1*) and TG241 (Δ*rcn2*, Δ*trp1*) were transformed with pCgACT-RCN1 and pCgACT-RCN2, respectively (Tables 1 and 3).

The Vcx1-dependent growth assay using a *S. cerevisiae* Δ*pmc1* mutant

To determine effects of overexpression of the *RCN* genes on calcineurin signaling, we performed the Vcx1-dependent growth assay using a *S. cerevisiae* Δ*pmc1* mutant as described previously (Kingsbury & Cunningham, 2000, Mehta*, et al.*, 2009). The open reading frames (ORFs) of *S. cerevisiae* and *C. glabrata RCN* genes were amplified from the genomic DNA of BY4742 and CBS138, respectively, and then inserted into the pYES2.1/V5-His-TOPO vector containing the galactose-inducible *GAL1* promoter (Invitrogen, Carlsbad, CA) to generate *RCN* gene-overexpression plasmids (Table 3). Growth of *S. cerevisiae* Δ*pmc1* mutants transformed with either empty vector or an *RCN* gene-overexpression plasmid was examined using dextrose and galactose plates in the presence and absence of 0.3 M $CaCl_2$. Plates were incubated at 30 °C for 48 h. The spot dilution test was performed twice on independent occasions.

Examination of antifungal tolerance of the constructed *C. glabrata* mutants

To examine growth of the *C. glabrata* strains in the presence of antifungal agents, a spot dilution test was performed as described previously (Miyazaki*, et al.*, 2010b). Briefly, logarithmic-phase cells grown in SC-trp broth were harvested and adjusted to 2×10^7 cells/ml. Serial 10-fold dilutions were then prepared, and 5 µl of each dilution was spotted onto SC-trp agar plates in the presence and absence of micafungin, tunicamycin and fluconazole. Plates

were incubated at 30 °C for 48 h. The spot dilution tests were repeated twice on independent occasions.

Quantitative real-time RT-PCR

To examine expression levels of the *YPS1* gene, logarithmic-phase cells grown in SC-trp broth (pH 5.5) were adjusted to 1×10^7 cells/ml and then further incubated in the presence of 0.03 μg/ml micafungin for up to 4 h. Total RNA was extracted every hour using a FastRNA Red Kit (Qbiogene, Carlsbad, CA). Quantitative real-time RT-PCR was performed as described previously (Saijo, *et al.*, 2010). Briefly, first strand cDNA was synthesized with a QuantiTect Reverse Transcription kit (Qiagen, Valencia, CA) from 1 μg of total RNA in a final volume of 20 μl, and 3 μl of resulting cDNA was then used as the template for individual PCR with gene-specific primers (Table 2), using a QuantiTect SYBR Green PCR kit (Qiagen). Quantitative real-time RT-PCR was performed in triplicate in a 96-well plate format, using a 7500 Real-Time PCR System (Applied Biosystems, Foster City, CA).

To examine expression levels of the *RCN* genes, total RNA was extracted from logarithmic-phase cultures (OD_{600} = 0.8) of each *C. glabrata* strain grown in SC-trp broth at 30 °C. cDNA synthesis and quantitative real-time RT-PCR were performed as described above. The mRNA abundance of the target gene was normalized to *ACT1* transcript levels. The real-time RT-PCR assays were repeated twice on independent occasions.

RESULTS AND DISCUSSION

Overexpression of *C. glabrata RCN1 and RCN2* inhibited calcineurin signaling in *S. cerevisiae*

The NCBI BLASTp search (http://www.ncbi.nlm.nih.gov/BLAST/) using *S. cerevisiae* Rcn1 and Rcn2 identified their *C. glabrata* orthologs: *C. glabrata* Rcn1 (NCBI accession number, XP_445961; Genolevures ID, CAGL0E06248g) showing 57.5% similarity with *S. cerevisiae* Rcn1 and *C. glabrata* Rcn2 (NCBI accession number, XP_447886; Genolevures ID, CAGL0J04158g) showing 54.6% similarity with *S. cerevisiae* Rcn2. The pairwise alignments of the deduced amino acid sequences of *C. glabrata* RCNs with their *S. cerevisiae* orthologs are presented in Fig. 1. The PxIxIT-like motif (consensus sequence [PG]x[IV]x[IVL][EDNHT]), which is typically found in RCNs, is important for mediating the inhibitory effects on calcineurin signaling because it allows these proteins to bind to the substrate-docking groove of calcineurin, and thus competes with other calcineurin substrates (Mehta*, et al.*, 2009). The PxIxIT-like motif was found in both *S. cerevisiae* and *C. glabrata* RCNs (Fig. 1): GAITID in *S. cerevisiae* Rcn1/173-178, GTITVN in *C. glabrata* Rcn1/161-166, PSITVN in *S. cerevisiae* Rcn2/256-261, and PSITVD in CgRcn2/222-227.

We next analyzed the inhibitory effects of RCNs on calcineurin signaling by the Vcx1-dependent growth assay using a *S. cerevisiae* Δ*pmc1* mutant as described previously (Kingsbury & Cunningham, 2000, Mehta*, et al.*, 2009). The rationale for this approach is represented schematically in Fig. 2a. In agreement with previous reports (Kingsbury & Cunningham, 2000, Mehta*, et al.*, 2009), overexpression of *S. cerevisiae RCN1* and *RCN2* rescued the growth of the Δ*pmc1* mutant in the presence of 0.3 M $CaCl_2$ (Fig. 2b). Under identical conditions, overexpression of *C. glabrata RCN1* and *RCN2* orthologs also displayed

similar effects. The results suggest that *S. cerevisiae* and *C. glabrata* RCNs have retained functional similarity regarding the inhibitory activity on calcineurin signaling.

Rcn1, but not Rcn2, is required for micafungin tolerance in *C. glabrata*

We next constructed deletion strains of *RCN1* and *RCN2* in *C. glabrata* and examined their mutant phenotypes. How calcineurin contributes to azole tolerance remains unknown, but our previous study demonstrated that calcineurin plays a role in tolerance to the β1,3-glucan synthesis inhibitor micafungin via the transcription factor Crz1 in *C. glabrata* (Miyazaki, *et al.*, 2010a). Therefore, we first investigated how the *C. glabrata RCN* mutants behave in the presence of micafungin. Deletion of *RCN1*, *CNB1* (a regulatory B subunit of calcineurin), or *CRZ1* resulted in decreased tolerance to micafungin, while reintroduction of the corresponding wild-type gene into the mutants recovered this phenotype (Fig. 3a). However, micafungin tolerance was not affected in the Δ*rcn2* deletant, and the Δ*rcn1* Δ*rcn2* double deletant did not display an additive phenotype compared with the Δ*rcn1* single deletant. These results suggest that calcineurin, Crz1, and Rcn1, but not Rcn2, are required for cell growth in the presence of micafungin in *C. glabrata*.

Rcn1, but not Rcn2, is required for activation of the calcineurin-Crz1 pathway in *C. glabrata*

Although the activity of calcineurin-Crz1 signaling can be measured by monitoring the expression levels of Crz1 target genes, neither a target of Crz1 nor the presence of a calcineurin-dependent response element (CDRE) has been reported in *C. glabrata*. Recently, we confirmed that the calcineurin-Crz1 pathway in *C. glabrata* regulates expression of the

YPS1 gene, which encodes a glycosylphosphatidylinositol-linked aspartyl protease required for cell wall integrity (Kaur, *et al.*, 2007), via putative CDREs in its promoter region (Miyazaki, *et al.*, 2011). Here, we therefore monitored changes of *YPS1* expression in response to micafungin treatment in the *C. glabrata* wild-type and mutant strains by quantitative real-time RT-PCR. As *YPS1* is a target gene of Crz1, *YPS1* expression was increased approximately fivefold in the *CRZ1*-overexpressing strain compared to that in the wild-type strain, even in the absence of micafungin (Fig. 3b). In the presence of micafungin, the expression level of *YPS1* was increased approximately fourfold in the wild-type and Δ*rcn2* deletion strains, but no induction was observed in the Δ*cnb1*, Δ*crz1*, Δ*rcn1*, and Δ*rcn1* Δ*rcn2* strains (Fig. 3b).

It has been suggested that RCNs function primarily as chaperones for the biosynthesis or recycling of calcineurin, leading to the stimulation of calcineurin signaling, but only when RCN proteins are expressed at physiological levels (Kingsbury & Cunningham, 2000, Mehta, *et al.*, 2009). Mehta et al. (Mehta, *et al.*, 2009) demonstrated that the LxxP, ExxP, and TxxP motifs and a GSK-3 phosphorylation site are required for the stimulatory activity of Rcn1 on calcineurin function in *S. cerevisiae*. In agreement with the observed mutant phenotypes, all three motifs and a putative GSK-3 phosphorylation site were present in *C. glabrata* Rcn1 (Fig. 3c), but none could be identified in Rcn2 of either *S. cerevisiae* or *C. glabrata*. Taken together, the results suggest that *C. glabrata* cells require Rcn1, but not Rcn2, to activate the calcineurin-Crz1 pathway in response to micafungin exposure.

Loss of Rcn1 impairs both Crz1–dependent and –independent signaling in *C. glabrata*

To further validate the observed functional differences between Rcn1 and Rcn2 in *C. glabrata*, the susceptibility of the mutants to other types of stress was also examined. In *C. glabrata*, although the Δ*rcn1* strain showed decreased tolerance to both tunicamycin and fluconazole, which primarily cause endoplasmic reticulum and cell membrane stresses, respectively, the Δ*rcn2* strain did not display these phenotypes (Fig. 4). Growth of the Δ*rcn1* Δ*rcn2* double deletant was similar to that of the Δ*rcn1* single deletant in the presence of tunicamycin and fluconazole, which was consistent with the findings of the micafungin susceptibility assay (Fig. 3a).

Both the Δ*rcn1* and Δ*cnb1* strains exhibited decreased tolerance to tunicamycin and fluconazole, but the additional deletion of *RCN1* in the Δ*cnb1* strain had no additive effect (Fig. 4). Although Crz1 is the only known downstream effector of calcineurin in pathogenic fungi, the Δ*crz1* mutant did not exhibit decreased tolerance to tunicamycin, but did have increased tolerance to fluconazole, as previously observed (Miyazaki, *et al.*, 2010a), suggesting that a Crz1-independent mechanism(s) is required for tolerance to these drugs in *C. glabrata*. Notably, the Δ*crz1* Δ*rcn1* double deletant exhibited drastically decreased tolerance to these drugs compared with the Δ*crz1* single deletant, showing growth defects similar to those of the Δ*cnb1* and Δ*rcn1* mutants. In contrast, the Δ*crz1* Δ*rcn2* double deletant displayed no additive phenotype compared with the Δ*crz1* single deletant (Fig. 4). The loss of *RCN1* and *RCN2* did not affect amphotericin B susceptibility (data not shown), consistent with our previous report that neither calcineurin nor Crz1 is involved in amphotericin B susceptibility in *C. glabrata* (Miyazaki, *et al.*, 2010a). In *C. albicans*, strains lacking either *CNB1* or *RCN1*

are highly susceptible to SDS (Reedy, *et al.*, 2009), but deletion of either or both of these genes in *C. glabrata* did not affect SDS tolerance (data not shown). It was determined that the Δ*rcn1* mutant phenocopied the Δ*cnb1* mutant, while the loss of *RCN1* in the Δ*cnb1* mutant had no additive effect on the Δ*cnb1* phenotypes observed in this study. Taken together, these results indicate that calcineurin requires Rcn1 to be fully functional; therefore loss of Rcn1 impairs both Crz1-dependent and –independent signaling in *C. glabrata*. Our results also suggest that Rcn1 plays a dominant regulatory role in calcineurin signaling and is unlikely to possess a calcineurin-independent function in *C. glabrata*.

Expression of *RCN2* is regulated by the calcineurin-Crz1 pathway in *C. glabrata*

Although overexpression of *C. glabrata RCN2* exerted inhibitory effects on calcineurin signaling, deletion of the gene conferred no phenotype in the settings tested in this study. Therefore, we performed real-time RT-PCR assays to check the expression levels of *RCN1* and *RCN2* in the *C. glabrata* wild-type and mutant strains (Fig. 5). The transcription of *RCN1* and *RCN2* was abolished in the strains lacking the corresponding gene, supporting that the gene deletion was successfully accomplished. Notably, the basal expression level of *RCN2*, but not *RCN1*, was suppressed in the strains that lacked either calcineurin, Crz1 or Rcn1. A genome-wide analysis in *S. cerevisiae* previously identified the consensus Crz1-binding site 5'-GNGGC(G/T)CA-3' and also revealed that the promoters of most calcineurin-dependent genes contain one to six copies of a core Crz1-binding sequence 5'-GNGGC(G/T)-3' (Yoshimoto, *et al.*, 2002). We found two putative Crz1-binding sites, 5'-GAGGCTTA-3' (-573 to -566) and 5'-GAGGCGAA-3' (-273 to -266), and another copy of the core Crz1-binding sequence, 5'-GAGGCT-3' (-805 to -800), within the 1-kb upstream region of *C.*

glabrata RCN2. In contrast, the sequence 5'-GNGGC(G/T)-3' was not present in the 1-kb upstream region of *C. glabrata RCN1*. Taken together, it is likely that calcineurin regulates *RCN2* expression via the Crz1 transcription factor in *C. glabrata*.

Concluding remarks

This is the first report characterizing the functions of RCNs in *C. glabrata*. The present study provides fundamental information to further understand how calcineurin is regulated in pathogenic fungi. The proposed *C. glabrata* calcineurin pathway is presented schematically in Fig. 6. While Rcn1 exerted both positive and negative effects on calcineurin signaling, Rcn2 appeared to lack enhancing effects on calcineurin function. *RCN2* was transcriptionally regulated by Crz1 and overexpression of *RCN2* inhibited calcineurin signaling, suggesting that Rcn2 functions as an endogenous feedback inhibitor of calcineurin signaling. Calcineurin is a key mediator of stress responses in pathogenic fungi, and inhibition of calcineurin function enhances the efficacy of existing antifungals, including fluconazole, even for the azole-resistant fungal pathogen *C. glabrata*. Inhibition of fungal calcineurin signaling by modulating endogenous regulators of calcineurin seems to be an attractive antifungal strategy, although its selective toxicity remains a critical issue. Further elucidation of this important signaling pathway will aid in the development of novel strategies for antifungal therapy.

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FIGURE LEGENDS

Fig. 1. The pairwise alignments of *C. glabrata* Rcn1 and Rcn2 with their *S. cerevisiae* orthologs. Sequence alignment was depicted by ClustalW alignment with MacVector software (version 11.0.2). The conserved PxIxIT-like motif (consensus sequence [PG]x[IV]x[IVL][EDNHT]) is underlined.

Fig. 2. Overexpression of *C. glabrata RCN1* and *RCN2* inhibits calcineurin signaling in *S. cerevisiae*. (a) Schematic representation of the rationale for the Vcx1-dependent growth assay to measure calcineurin inhibition. The activity of a vacuole Ca^{2+} transporter, either Pmc1 or Vcx1, is necessary for cell growth in high-Ca^{2+} environments in *S. cerevisiae*. Functional calcineurin induces the expression of *PMC1*, but simultaneously inhibits Vcx1 function (Cunningham & Fink, 1994, Cunningham & Fink, 1996); thus, the loss of Pmc1 is lethal to cells under high-Ca^{2+} conditions. However, the inhibition of calcineurin leads to Vcx1 activation, and Δ*pmc1* mutant cells can survive calcium stress. (b) Overexpression of the *S. cerevisiae* and *C. glabrata RCN* genes rescues the viability of *S. cerevisiae* Δ*pmc1* mutants in high-calcium conditions. *S. cerevisiae RCN1*, *RCN2*, and their *C. glabrata* orthologs were expressed under control of the inducible *GAL1* promoter. *S. cerevisiae* Δ*pmc1* strains transformed with either empty vector or an overexpression plasmid were adjusted to 2×10^7 cells/ml, and 5 μl of serial 10-fold dilutions was then spotted onto yeast peptone dextrose (YPD) and yeast peptone galactose (YPG) plates in the presence and absence of 0.3 M calcium chloride. Plates were incubated at 30 °C for 48 h. Representative results of two independent experiments are shown.

Fig. 3. Rcn1, but not Rcn2, is required for cell growth and activation of the calcineurin-Crz1 pathway in the presence of micafungin. (a) *C. glabrata* calcineurin (Cnb1), Crz1, and Rcn1, but not Rcn2, are required for cell growth in the presence of micafungin. The indicated *C. glabrata* deletion mutants were transformed with either empty vector or a plasmid containing the corresponding wild-type gene. Logarithmic-phase cells were adjusted to 2×10^7 cells/ml, and 5 μl of serial 10-fold dilutions was then spotted onto synthetic complete medium lacking tryptophan (Sc-trp) in the presence and absence of 0.013 μg/ml micafungin. Plates were incubated at 30 °C for 48 h. Representative results of three independent experiments are shown. (b) Time-course analysis of *YPS1* expression, a downstream target of the calcineurin-Crz1 pathway, in the *C. glabrata* wild-type and mutant strains. Logarithmic-phase cells (1×10^7 cells/ml) were incubated at 30 °C in the presence of 0.03 μg/ml micafungin. Total RNA was extracted at the indicated time points and *YPS1* mRNA was measured by quantitative real-time RT-PCR. Results represent the average of two independent experiments. Error bars are standard deviations. *C. glabrata* strains: wild-type, TG12; Δ*rcn1*, TG232; Δ*rcn1* + *RCN1*, TG233; Δ*rcn2*, TG242; Δ*rcn2* + *RCN2*, TG243; Δ*cnb1*, TG162; Δ*cnb1* + *CNB1*, TG163; Δ*crz1*, TG172; Δ*crz1* + *CRZ1*, TG173; Δ*rcn1* Δ*rcn2*, TG2324; and *CRZ1* OE (*CRZ1* overexpression), TG173. (c) The conserved motifs required for the stimulatory effects of Rcn1 on calcineurin signaling. Alignment of the putative amino acid sequences of several Rcn1 orthologs was generated using the MacVector program. The amino acid position of each gene is indicated. The SP-linker domain contains the GSK-3 phosphorylation site (S;*) and the LxxP and ExxP motifs. GenBank accession numbers: *Saccharomyces cerevisiae* Rcn1, NP012763; *Candida glabrata* Rcn1, CAG58880; *Candida albicans* Rcn1-1, XP714329; *Cryptococcus neoformans* Cbp1, AAU11489; *Schizosaccharomyces pombe* Rcn1, Q09791;

Drosophila melanogaster RCN, AAD33987; *Caenorhabditis elegans* RCN-1, P53806; and *Homo sapiens* RCAN1, AAH02864.

Fig. 4. Growth of the *C. glabrata* mutants in the presence of tunicamycin and fluconazole. Logarithmic-phase cells were adjusted to 2×10^7 cells/ml, and 5 μl of serial 10-fold dilutions was then spotted onto SC-trp agar plates in the presence and absence of tunicamycin and fluconazole at the indicated concentrations. Plates were incubated at 30 °C for 48 h. Representative results of three independent experiments are shown. *C. glabrata* strains: wild-type, TG12; Δ*rcn1*, TG232; Δ*rcn1* + *RCN1*, TG233; Δ*rcn2*, TG242; Δ*rcn2* + *RCN2*, TG243; Δ*rcn1* Δ*rcn2*, TG2324; Δ*cnb1*, TG162; Δ*cnb1* Δ*rcn1*, TG1623; Δ*cnb1* Δ*rcn2*, TG1624; Δ*crz1*, TG172; Δ*crz1* Δ*rcn1*, TG1723; and Δ*crz1* Δ*rcn2*, TG1724.

Fig. 5. The basal expression levels of *RCN1* and *RCN2* in the *C. glabrata* wild-type and mutant strains. Total RNA was extracted from logarithmic-phase cultures and cDNA was synthesized as described in Materials and methods. The expression levels of *RCN1* and *RCN2* were measured by quantitative real-time RT-PCR using gene-specific primers. The results are expressed relative to the values in the wild-type strain. Error bars represent standard deviations from the means of three independent experiments. *C. glabrata* strains are as described in the legend to Fig. 4.

Fig. 6. Schematic representation of the proposed calcineurin-signaling pathway in *C. glabrata*. Calcineurin activated by the calmodulin-Ca^{2+} complex dephosophorylates the transcription factor Crz1 in the cytoplasm, leading to its nuclear migration and the subsequent activation of various stress responses. Crz1 transcriptionally regulates Rcn2 that, when overexpressed, subsequently inhibits calcineurin signaling, suggesting an endogenous feedback inhibition mechanism of calcineurin signaling. Rcn1 exerts both positive and negative effects on calcineurin signaling. *C. glabrata* calcineurin also regulates an unknown downstream target that is involved in stress response independently of Crz1.

Fig. 1.

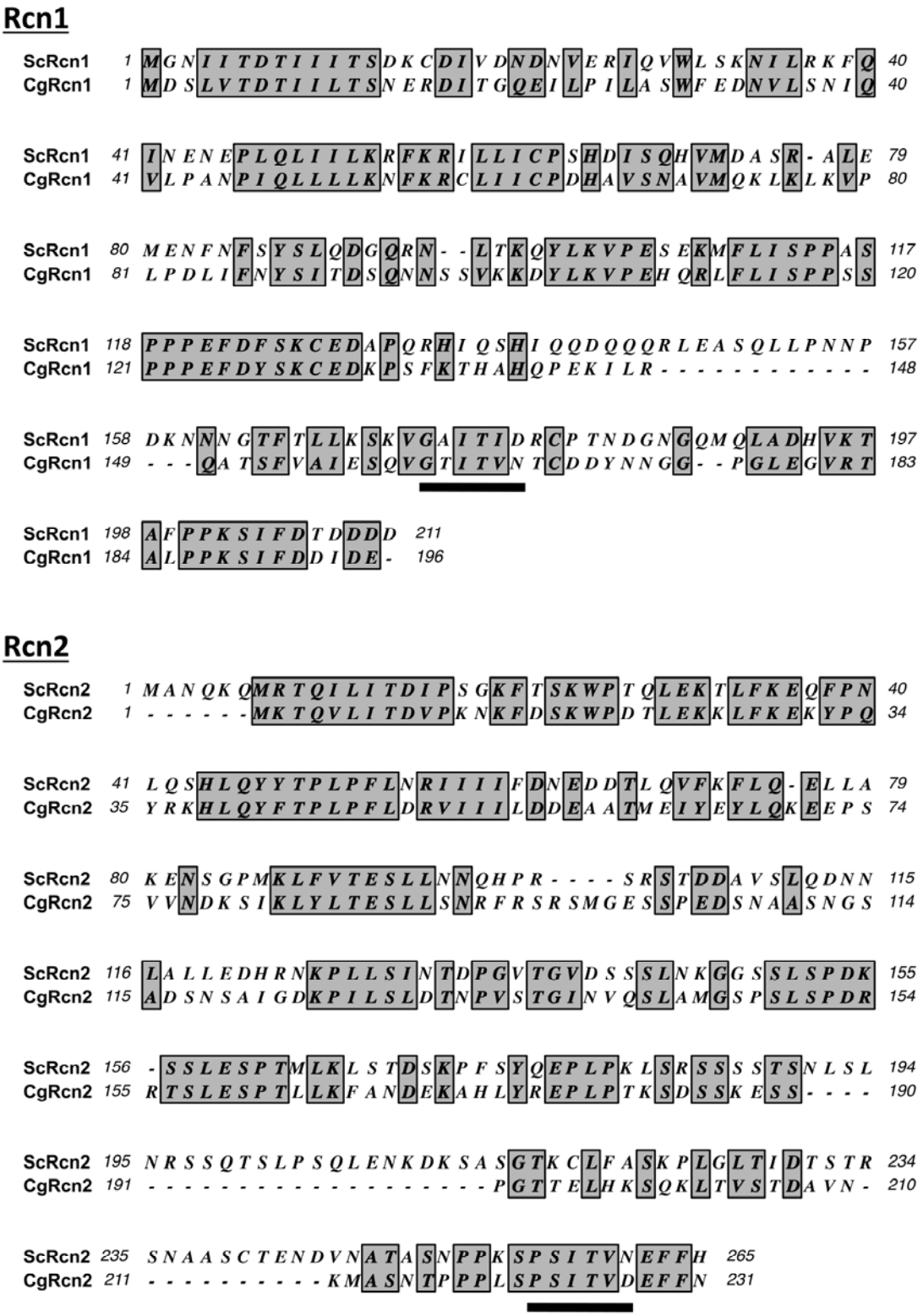

Rcn1
ScRcn1 1 MGNIITDTIIITSDKCDIVDNDNVERIQVWLSKNILRKFQ 40
CgRcn1 1 MDSLVTDTIILTSNERDITGQEILPILASWFEDNVLSNIQ 40
ScRcn1 41 INENEPLQLIILKRFKRILLICPSHDISQHVMDASR-ALE 79
CgRcn1 41 VLPANPIQLLLLKNFKRCLIICPDHAVSNAVMQKLKLKVP 80
ScRcn1 80 MENFNFSYSLQDGQRN--LTKQYLKVPESEKMFLISPPAS 117
CgRcn1 81 LPDLIFNYSITDSQNNSSVKKDYLKVPEHQRLFLISPPSS 120
ScRcn1 118 PPPEFDFSKCEDAPQRHIQSHIQQDQQQRLEASQLLPNNP 157
CgRcn1 121 PPPEFDYSKCEDKPSFKTHAHQPEKILR------------ 148
ScRcn1 158 DKNNNGTFTLLKSKVGAITIDRCPTNDGNGQMQLADHVKT 197
CgRcn1 149 ---QATSFVAIESQVGTITVNTCDDYNNGG--PGLEGVRT 183
ScRcn1 198 AFPPKSIFDTDDDD 211
CgRcn1 184 ALPPKSIFDDIDE- 196
Rcn2
ScRcn2 1 MANQKQMRTQILITDIPSGKFTSKWPTQLEKTLFKEQFPN 40
CgRcn2 1 ------MKTQVLITDVPKNKFDSKWPDTLEKKLFKEKYPQ 34
ScRcn2 41 LQSHLQYYTPLPFLNRIIIIFDNEDDTLQVFKFLQ-ELLA 79
CgRcn2 35 YRKHLQYFTPLPFLDRVIIILDDEAATMEIYEYLQKEEPS 74
ScRcn2 80 KENSGPMKLFVTESLLNNQHPR----SRSTDDAVSLQDNN 115
CgRcn2 75 VVNDKSIKLYLTESLLSNRFRSRSMGESSPEDSNAASNGS 114
ScRcn2 116 LALLEDHRNKPLLSINTDPGVTGVDSSSLNKGGSSLSPDK 155
CgRcn2 115 ADSNSAIGDKPILSLDTNPVSTGINVQSLAMGSPSLSPDR 154
ScRcn2 156 -SSLESPTMLKLSTDSKPFSYQEPLPKLSRSSSSTSNLSL 194
CgRcn2 155 RTSLESPTLLKFANDEKAHLYREPLPTKSDSSKESS---- 190
ScRcn2 195 NRSSQTSLPSQLENKDKSASGTKCLFASKPLGLTIDTSTR 234
CgRcn2 191 ----------------------PGTTELHKSQKLTVSTDAVN- 210
ScRcn2 235 SNAASCTENDVNATASNPPKSPSITVNEFFH 265
CgRcn2 211 ----------KMASNTPPLSPSITVDEFFN 231

Fig. 2.

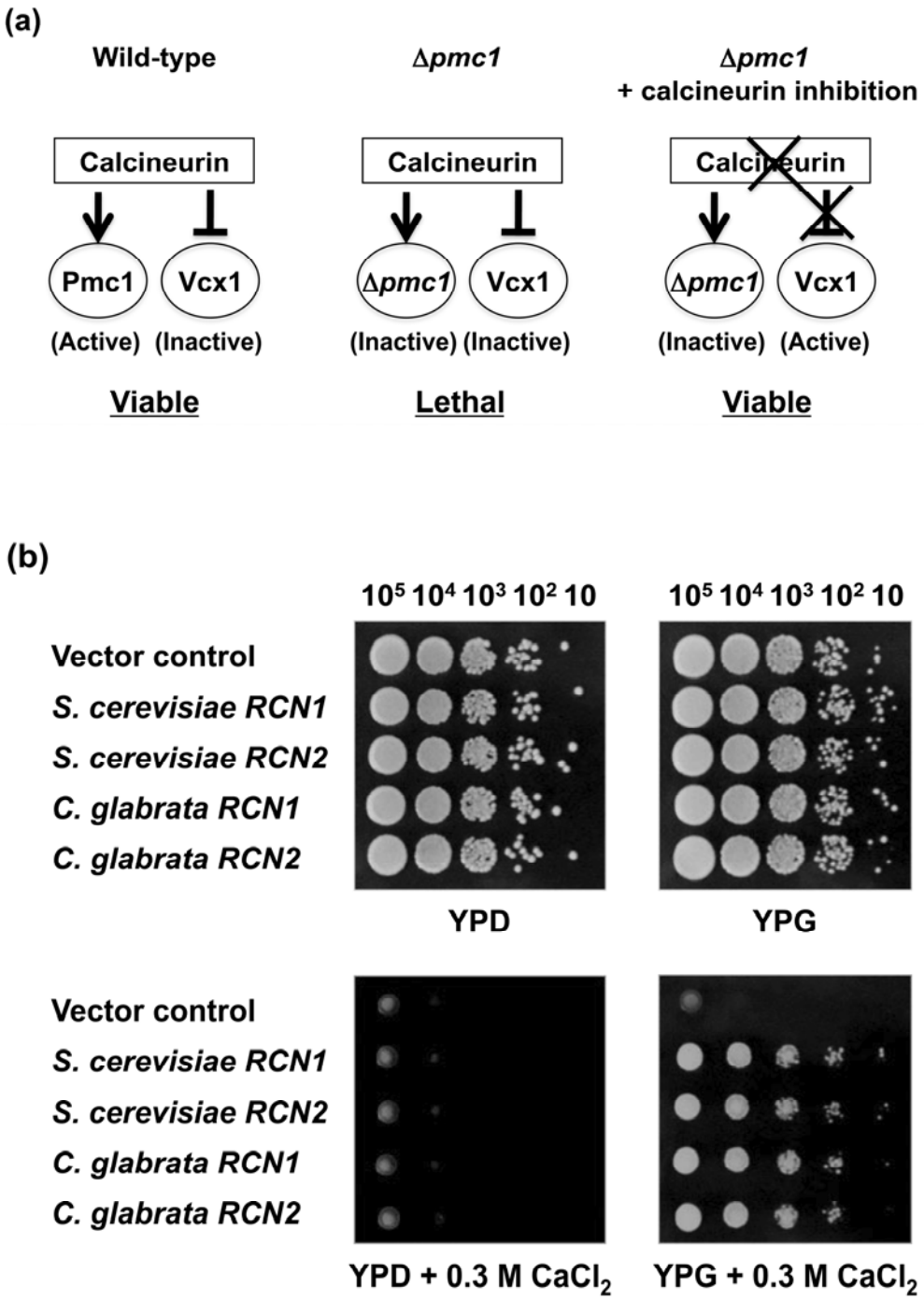
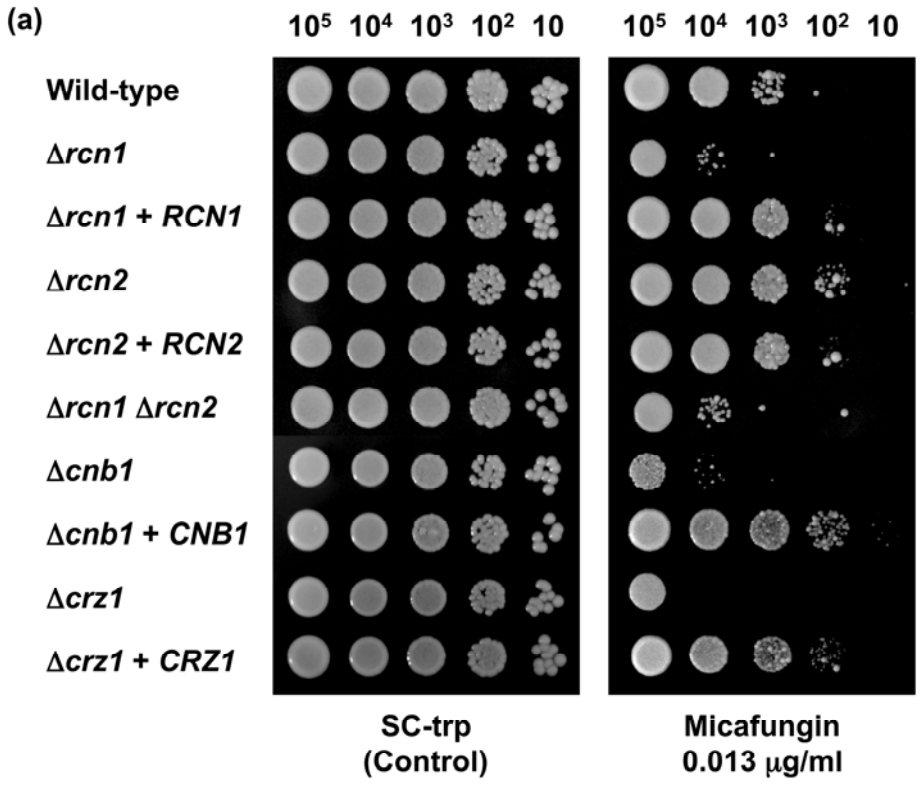

(a)
Wild-type
Δpmc1
Δpmc1
+ calcineurin inhibition
Calcineurin
Calcineurin
Calcineurin
Pmc1
Vcx1
Δpmc1
Vcx1
Δpmc1
Vcx1
(Active) (Inactive)
(Inactive) (Inactive)
(Inactive) (Active)
Viable
Lethal
Viable
(b)
10⁵ 10⁴ 10³ 10² 10
10⁵ 10⁴ 10³ 10² 10
Vector control
S. cerevisiae RCN1
S. cerevisiae RCN2
C. glabrata RCN1
C. glabrata RCN2
YPD
YPG
Vector control
S. cerevisiae RCN1
S. cerevisiae RCN2
C. glabrata RCN1
C. glabrata RCN2
YPD + 0.3 M CaCl2
YPG + 0.3 M CaCl2

Fig. 3.


(a)
10⁵ 10⁴ 10³ 10² 10
10⁵ 10⁴ 10³ 10² 10
Wild-type
Δrcn1
Δrcn1 + RCN1
Δrcn2
Δrcn2 + RCN2
Δrcn1 Δrcn2
Δcnb1
Δcnb1 + CNB1
Δcrz1
Δcrz1 + CRZ1
SC-trp
(Control)
Micafungin
0.013 μg/ml

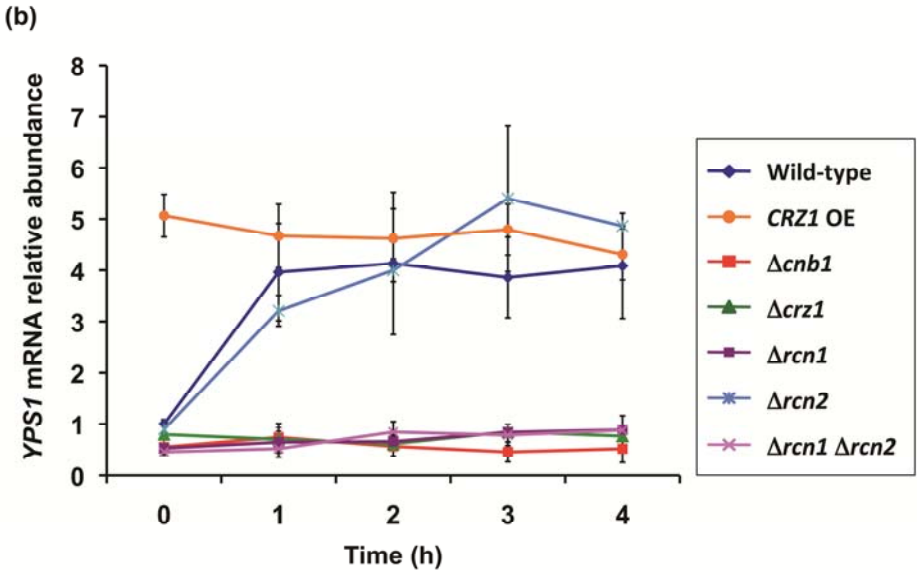

(b)
YPS1 mRNA relative abundance
0
1
2
3
4
5
6
7
8
Time (h)
0
1
2
3
4
Wild-type
CRZ1 OE
Δcnb1
Δcrz1
Δrcn1
Δrcn2
Δrcn1 Δrcn2

Fig. 3.

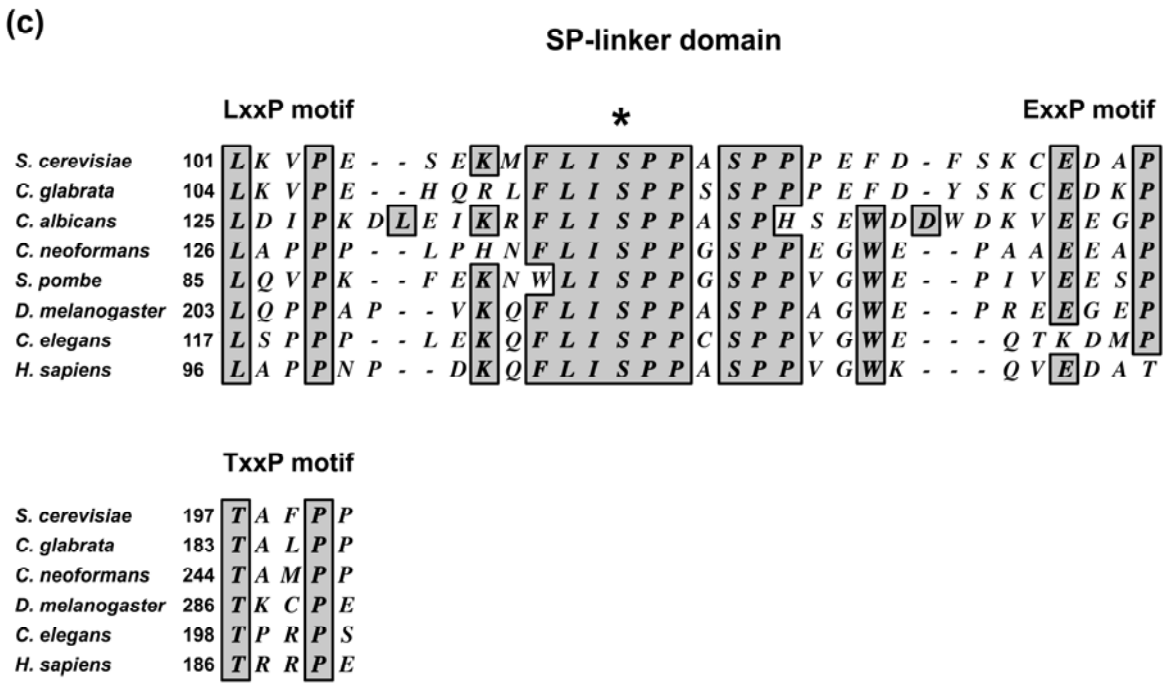

(c)
SP-linker domain
LxxP motif
*
ExxP motif
S. cerevisiae 101 L K V P E - - S E K M F L I S P P A S P P P E F D - F S K C E D A P
C. glabrata 104 L K V P E - - H Q R L F L I S P P S S P P P E F D - Y S K C E D K P
C. albicans 125 L D I P K D L E I K R F L I S P P A S P H S E W D D W D K V E E G P
C. neoformans 126 L A P P P - - L P H N F L I S P P G S P P E G W E - - P A A E E A P
S. pombe 85 L Q V P K - - F E K N W L I S P P G S P P V G W E - - P I V E E S P
D. melanogaster 203 L Q P P A P - - V K Q F L I S P P A S P P A G W E - - P R E E G E P
C. elegans 117 L S P P P - - L E K Q F L I S P P C S P P V G W E - - - Q T K D M P
H. sapiens 96 L A P P N P - - D K Q F L I S P P A S P P V G W K - - - Q V E D A T
TxxP motif
S. cerevisiae 197 T A F P P
C. glabrata 183 T A L P P
C. neoformans 244 T A M P P
D. melanogaster 286 T K C P E
C. elegans 198 T P R P S
H. sapiens 186 T R R P E

Fig. 4.

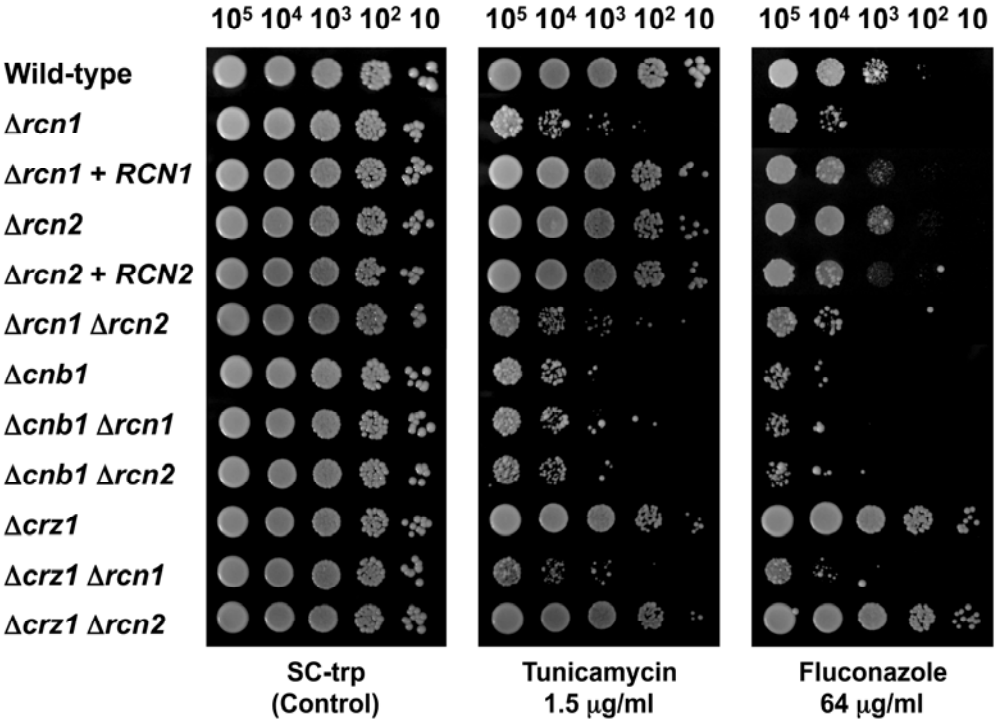

10⁵ 10⁴ 10³ 10² 10
10⁵ 10⁴ 10³ 10² 10
10⁵ 10⁴ 10³ 10² 10
Wild-type
Δrcn1
Δrcn1 + RCN1
Δrcn2
Δrcn2 + RCN2
Δrcn1 Δrcn2
Δcnb1
Δcnb1 Δrcn1
Δcnb1 Δrcn2
Δcrz1
Δcrz1 Δrcn1
Δcrz1 Δrcn2
SC-trp
(Control)
Tunicamycin
1.5 μg/ml
Fluconazole
64 μg/ml

Fig. 5.

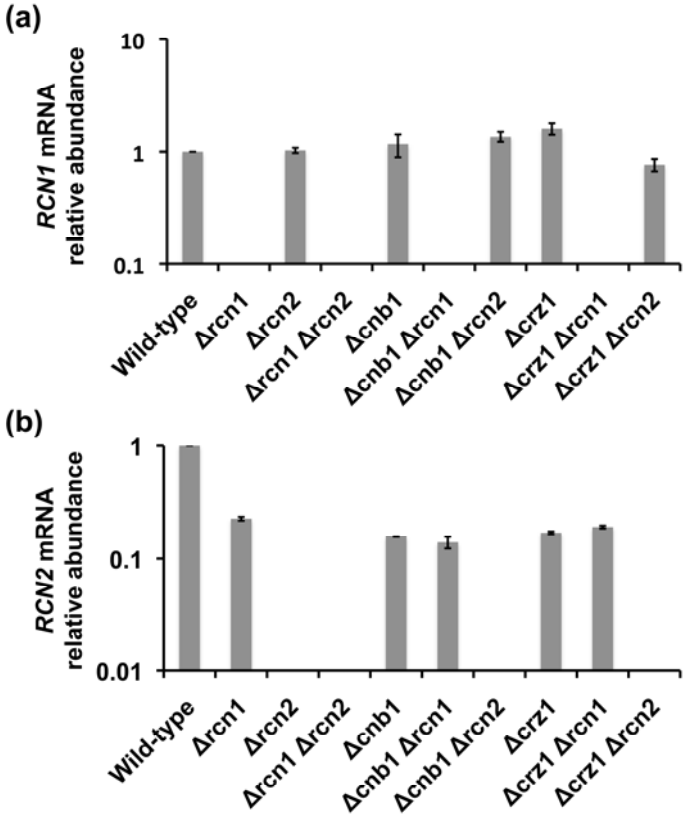

(a)
RCN1 mRNA relative abundance
10
1
0.1
Wild-type
Δrcn1
Δrcn2
Δrcn1 Δrcn2
Δcnb1
Δcnb1 Δrcn1
Δcnb1 Δrcn2
Δcrz1
Δcrz1 Δrcn1
Δcrz1 Δrcn2
(b)
RCN2 mRNA relative abundance
1
0.1
0.01
Wild-type
Δrcn1
Δrcn2
Δrcn1 Δrcn2
Δcnb1
Δcnb1 Δrcn1
Δcnb1 Δrcn2
Δcrz1
Δcrz1 Δrcn1
Δcrz1 Δrcn2

Fig. 6.

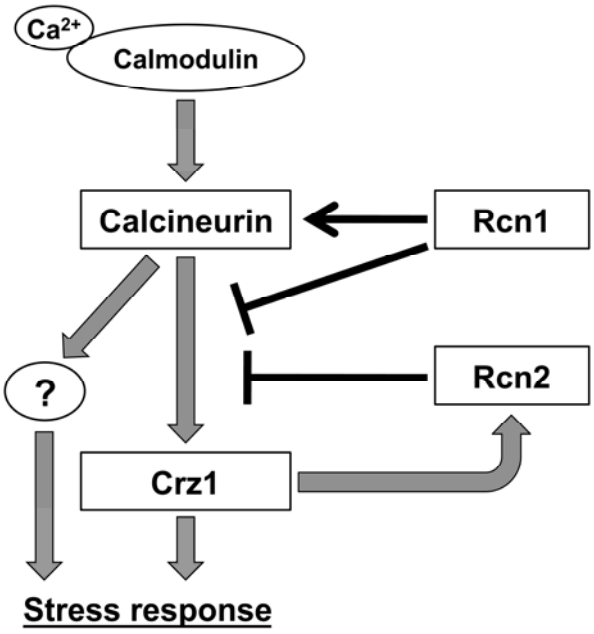

Ca2+
Calmodulin
Calcineurin
Rcn1
Rcn2
?
Crz1
Stress response

Table 1. Strains used in this study

Strain	Genotype or description	Reference or source
S. cerevisiae		
BY4742	MATα Δ*his3*, Δ*leu2*, Δ*lys2*, Δ*ura3*	(Winzeler, *et al.*, 1999)
SCPM1	As BY4742, Δ*pmc1*::*KAN*	Open Biosystems
SCPM1-vec	SCPM1 containing pYES2.1	This study
SCPM1-S1	SCPM1 containing pYES-SCR1	This study
SCPM1-S2	SCPM1 containing pYES-SCR2	This study
SCPM1-C1	SCPM1 containing pYES-CGR1	This study
SCPM1-C2	SCPM1 containing pYES-CGR2	This study
C. glabrata		
CBS138	Wild-type	(Dujon, *et al.*, 2004)
2001T	Δ*trp1*	(Kitada, *et al.*, 1995)
2001HT	Δ*his3*, Δ*trp1*	(Kitada, *et al.*, 1995)
TG12	2001T containing pCgACT	This study
TG161	Δ*cnb1*::*HIS3*, Δ*trp1* (made from 2001HT)	(Miyazaki, *et al.*, 2010a)
TG162	TG161 containing pCgACT-P	(Miyazaki, *et al.*, 2010a)
TG163	TG161 containing pCgACT-PNB	(Miyazaki, *et al.*, 2010a)
TG171	Δ*crz1*::*HIS3*, Δ*trp1* (made from 2001HT)	(Miyazaki, *et al.*, 2010a)
TG172	TG171 containing pCgACT-P	(Miyazaki, *et al.*, 2010a)
TG173	TG171 containing pCgACT-PRZ	(Miyazaki, *et al.*, 2010a)
TG231	Δ*rcn1*::*HIS3*, Δ*trp1* (made from 2001HT)	This study
TG232	TG231 containing pCgACT	This study
TG233	TG231 containing pCgACT-RCN1	This study
TG241	Δ*rcn2*::*HIS3*, Δ*trp1* (made from 2001HT)	This study
TG242	TG241 containing pCgACT	This study
TG243	TG241 containing pCgACT-RCN2	This study
TG1623	Δ*cnb1*::*HIS3*, Δ*rcn1*::*TRP1*	This study
TG1624	Δ*cnb1*::*HIS3*, Δ*rcn2*::*TRP1*	This study
TG1723	Δ*crz1*::*HIS3*, Δ*rcn1*::*TRP1*	This study
TG1724	Δ*crz1*::*HIS3*, Δ*rcn2*::*TRP1*	This study
TG2324	Δ*rcn1*::*TRP1*, Δ*rcn2*::*HIS3*	This study

Table 2. Primers used in this study

Primer [a]	**Target gene**	**Sequence (5'– 3')** [b]
For gene deletion		
CgRCN1 100-F	*C. glabrata RCN1*	*CCTACCCAGTATGGGTGATGAGCAAGTTATG AATTGTAATGTGACATTAAAGTTATAAGGTCC CTAATGTAGTATATTTATTGTCCAGTTTTGAGA GGA***TAATACGACTCACTATAGGGC**
CgRCN1 100-R	*C. glabrata RCN1*	*GTAGTCTTCCCATCCCATTATGTTACTGAATTT GAATACCGAAAACCGTTTTCGACAGTTGCTGC TTCAATCTAGCAATATCTATATCACTTTATCTG T***CGCTCTAGAACTAGTGGATCC**
CgRCN2 100-F	*C. glabrata RCN2*	*GGCGTATAAGGTATACGTGCATATACATACAG AAGTAGATAATACGTCCAGTACACAGACATCA CACACAGACATAGACACAGACACGCAGATAT AGCA***TAATACGACTCACTATAGGGC**
CgRCN2 100-R	*C. glabrata RCN2*	*CCGTGTGCGTATATATAATTATGCATTCTAGC AACTGCTAACGTTTTTCCAATATGAGGTTCAA AAAAGACTATAACTAGCTTCATAGAAGCAATC CCC***CGCTCTAGAACTAGTGGATCC**
For gene cloning		
ScRCN1-F	*S. cerevisiae RCN1*	ATGGGTAATATTATAACGGATACG
ScRCN1-R	*S. cerevisiae RCN1*	AAGCCAACAAATCGCCTCGC
ScRCN2-F	*S. cerevisiae RCN2*	ATGGCAAACCAAAAGCAAATGA
ScRCN2-R	*S. cerevisiae RCN2*	TGGAAAGAGGACGAAAGTGTGC
CgRCN1-F	*C. glabrata RCN1*	ATGGATAGTCTTGTCACTGATACG
CgRCN1-F1	*C. glabrata RCN1*	CGGGATCCCCGACGCAGTATTCTTGTTC
CgRCN1-R	*C. glabrata RCN1*	CGCATTATGACAGTTCAACG
CgRCN1-R1	*C. glabrata RCN1*	CCGCTCGAGCGCATTATGACAGTTCAACG
CgRCN2-F	*C. glabrata RCN2*	ATGAAGACACAGGTATTGATTACGG
CgRCN2-F1	*C. glabrata RCN2*	CGGGATCCCAGTAATCTAATCAGGGCAGC
CgRCN2-R	*C. glabrata RCN2*	GCAACTGCTAACGTTTTTCCA
CgRCN2-R1	*C. glabrata RCN2*	CCGCTCGAGGCAACTGCTAACGTTTTTCCA

For real-time RT-PCR		
CgYPS1-F1281	*C. glabrata YPS1*	CGGATTCCACATCAACGCTC
CgYPS1-R1436	*C. glabrata YPS1*	TCGTAGTTTTCCAGGTCGTAGACG
CgRCN1-F129	*C. glabrata RCN1*	GGCAAATCCGATACAACTGTTAC
CgRCN1-R364	*C. glabrata RCN1*	GTGGGGAGGAAGGTGGTG
CgRCN2-F219	*C. glabrata RCN2*	CAGTGTGGTCAATGATAAGAGC
CgRCN2-R485	*C. glabrata RCN2*	GTTGGCGATTCAAGAGACG
CgACT1-F163	*C. glabrata ACT1*	GGTATGGGTCAAAAGGACTCTTACG
CgACT1-R305	*C. glabrata ACT1*	TCGTTGTAGAAAGTGTGATGCCAG

[a] "F" and "R" indicate forward and reverse primers, respectively.

[b] Sequences homologous to flanking regions of the target ORF are shown in italics. Sequences shown in boldface are present in pBSK-HIS and pBSK-TRP. Restriction sites are underlined.

Table 3. Plasmids used in this study

Plasmid	Description	Reference or source
pYES2.1	pYES2.1/V5-His-TOPO (2μ plasmid containing *GAL1* promoter, *CYC1* terminator, ampicillin resistance gene and *URA3*)	Invitrogen
pYES-SCR1	A 660-bp PCR fragment (primers ScRCN1-F and ScRCN1-R) containing the *S. cerevisiae RCN1* ORF was cloned into pYES2.1.	This study
pYES-SCR2	A 1084-bp PCR fragment (primers ScRCN2-F and ScRCN2-R) containing the *S. cerevisiae RCN2* ORF was cloned into pYES2.1.	This study
pYES-CGR1	A 672-bp PCR fragment (primers CgRCN1-F and CgRCN1-R) containing the *C. glabrata RCN1* ORF was cloned into pYES2.1.	This study
pYES-CGR2	A 774-bp PCR fragment (primers CgRCN2-F and CgRCN2-R) containing the *C. glabrata RCN2* ORF was cloned into pYES2.1.	This study
pBSK-HIS	pBluescript II SK+ (Stratagene, La Jolla, CA) containing *C. glabrata HIS3* at the XhoI site.	(Miyazaki, *et al.*, 2010b)
pBSK-TRP	A 1-kb XhoI fragment containing *C. glabrata TRP1* was excised from pCgACT and inserted into the XhoI site of pBluescript II SK+ (Stratagene).	This study
pCgACT	*C. glabrata* centromere-based plasmid containing autonomously replicating sequence and *C. glabrata TRP1*	(Kitada, *et al.*, 1996)
pCgACT-RCN1	A 1340-bp PCR fragment (primers CgRCN1-F1 and CgRCN1-R1) containing *C. glabrata RCN1* was inserted into the BamHI-XhoI site of pCgACT	This study
pCgACT-RCN2	A 580-bp PCR fragment (primers CgRCN2-F1 and CgRCN2-R1) containing *C. glabrata RCN2* was inserted into the BamHI-XhoI site of pCgACT	This study