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Elucidating the *Candida albicans* calcineurin signaling cascade controlling stress response and virulence

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Abstract

The protein phosphatase calcineurin is a key mediator of virulence and antifungal susceptibility of multiple fungal pathogens including *Candida albicans*, *Cryptococcus neoformans*, and *Aspergillus fumigatus*, and has clinical potential as a therapeutic target to increase the efficacy of the current antifungal armamentarium. Despite the importance of this signaling pathway, few components of the calcineurin-signaling pathway are known in *C. albicans.* Here we identified and analyzed additional components of the *C. albicans* calcineurin cascade, including the *RCN1* (Regulator of Calcineurin1), *MID1*, and *CCH1* genes, which mediate calcineurin functions in other species. When heterologous expressed in *S. cerevisiae*, *C. albicans* Rcn1 inhibited calcineurin function. Although *rcn1/rcn1*, *mid1/mid1*, and *cch1/cch1* mutant strains share some phenotypes with calcineurin mutants, they do not completely recapitulate the phenotypes of a calcineurin mutant strain. These studies extend our understanding of the *C. albicans* calcineurin signaling cascade and its host-niche specific role in virulence.

Keywords

Candida albicans; calcineurin; Rcn1; Mid1; Cch1; fungal pathogenesis; fluconazole

Introduction

Calcineurin is a calcium, calmodulin-dependent serine-threonine specific protein phosphatase that is highly conserved from yeast to humans and mediates many important cellular processes (Hemenway and Heitman, 1999). In mammalian cells, calcineurin is involved in cardiac muscle differentiation (Chin et al., 1998; Kramer et al., 2003; Parsons et al., 2007), memory (Mansuy et al., 1998; Weitlauf and Winder, 2001), T-cell activation (Clipstone and Crabtree, 1992), and apoptosis (Krebs, 1998; Saito et al., 2000; Shibasaki and McKeon, 1995; Wang et al., 1999). The immunosuppressants FK506 and Cyclosporin A (CsA) exert their effect by entering cells and binding to an immunophilin protein partner (FKBP12 for FK506, and Cyclophilin A for

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CsA) (Cardenas et al., 1994; Cardenas et al., 1995; Clipstone et al., 1994; Ho et al., 1996). This protein-drug complex subsequently binds calcineurin and inhibits its activity and functions. In human T-cells, calcineurin activates a transcription factor (NF-AT), which promotes the expression of cytokines and T-cell proliferation (Crabtree, 1999). Due to the highly conserved nature of calcineurin, it was subsequently found that FK506 and CsA can inhibit not only mammalian calcineurin, but also fungal calcineurin (Blankenship et al., 2003a; Breuder et al., 1994; Foor et al., 1992; Nakamura et al., 1993; Steinbach et al., 2007).

Candida spp. are normal components of the human microbiota; however, under conditions of immunosuppression or altered host defenses these commensals have the ability to cause serious mucocutaneous and systemic disease (Odds, 1988). Diseases due to *Candida spp.* manifest a variety of clinical manifestations, ranging from mucocutaneous infections mouth (thrush), esophagus, and vagina to life-threatening systemic infections, where *Candida spp.* enter the bloodstream and disseminate throughout the body to infiltrat target organs (Edwards, 1991). Although *C. albicans* has historically accounted for the majority of candidal infections, following the introduction of the antifungal fluconazole numerous other species have increased in prevalence including *C. glabrata, C. parapsilosis, C. tropicalis*, and *C. krusei* (Hazen, 1995; Krcmery and Barnes, 2002; Merz et al., 1986; Nguyen et al., 1996; Pfaller et al., 1998). Despite intensive drug discovery efforts, there are still only 3 classes of antifungal drugs that are available to treat serious fungal infections, Amphotericin B, azoles and echinocandins. Additionally, despite their widespread usage the azoles are fungistatic rather than fungicidal. Thus, there is need for new strategies and therapeutics to combat fungal infections.

Interestingly, the combination of calcineurin inhibitors and the normally fungistatic antifungal fluconazole result in potent killing of *C. albicans*, as well as other more drug resistant species such as *C. glabrata* (Cruz et al., 2002; Marchetti et al., 2000; Onyewu et al., 2003). Additionally, *C. albicans* calcineurin mutants have attenuated virulence in murine models of systemic infection (Bader et al., 2003; Blankenship et al., 2003b), and have faster rates of disease resolution in murine keratitis models (Onyewu et al., 2006). The attenuated virulence of *C. albicans* calcineurin mutants in systemic disease is attributable to the inability of these strains to withstand the calcium stress imposed by serum and thus survive transit through the bloodstream (Blankenship and Heitman, 2005). However, the direct role of calcineurin in *C. albicans* virulence appears to be host niche-specific as there was no virulence defect seen in either a vaginal or a pulmonary model of infection (Bader et al., 2006). Thus, calcineurin inhibitors have two potential mechanisms of action in the clinic: 1) as single agents in cases of disseminated disease or ocular infections to directly impair survival of the yeasts, or 2) as combination therapy to enhance the efficacy of current antifungal therapies. However, the immunosuppressive nature of calcineurin inhibitors limits their use in systemic therapy. Therefore, we were interested in further characterizing the calcineurin signaling cascade to learn more about this important stress response pathway. We also wanted to elucidate other components that could serve as alternative drug targets that would circumvent the immunosuppressive effects of inhibiting calcineurin.

In *C. albicans*, calcineurin is required for cells to survive stressors such as high cations (Li^+, Na^+, and Ca^{2+}), antifungal drug treatment (azoles), and the host bloodstream (Bader et al., 2003; Blankenship and Heitman, 2005; Blankenship et al., 2003b; Sanglard et al., 2003). Based upon homology with *S. cerevisiae* Crz1, the downstream transcription factor Crz1 (Cyert, 2003) was previously identified in *C. albicans*, and shown to shuttle into the nucleus in a calcineurin-dependent manner (Karababa et al., 2006). However, phenotypic analysis of *crz1/crz1* strains only partially recapitulated a calcineurin mutant phenotype. Although *crz1/crz1* mutant strains are sensitive to cations and membrane stresses, they exhibited an intermediate phenotype compared with calcineurin mutants (Karababa et al., 2006; Onyewu et al., 2004; Santos and de Larrinoa, 2005). Microarray studies have suggested that Crz1 is the primary

mediator of the calcineurin-dependent transcriptional response (Karababa et al., 2006). As a first step towards elucidating other potential genes in the *C. albicans* calcineurin pathway, we took a candidate gene approach based on analogous signaling pathways in *S. cerevisiae.*

Few proteins are known that are direct binding partners of calcineurin; these include calmodulin, transcription factors (Crz1, *C. albicans;* Crz1/Tcn1 *S. cerevisiae;* NF-AT, mammalian cells), and the RCAN family of proteins (Beals et al., 1997; Cyert, 2003; Davies et al., 2007; Hilioti and Cunningham, 2003; Karababa et al., 2006; Klee et al., 1979; Matheos et al., 1997; Onyewu et al., 2004; Santos and de Larrinoa, 2005; Stathopoulos and Cyert, 1997). Members of the RCAN family have been identified in species including *S. cerevisiae* (Rcn1), *C. neoformans* (Cbp1), and humans (DSCR1/MCIP1) based upon a conserved FLSPPxSP domain (Davies et al., 2007; Gorlach et al., 2000; Hilioti and Cunningham, 2003; Strippoli et al., 2000a). The function of these proteins has been best explored in *S. cerevisiae* where they exert both positive and negative effects on calcineurin function. Rcn1 binds calcineurin and inhibits its function. However, upon phosphorylation by a GSK3 kinase Rcn1 is degraded thereby relieving calcineurin inhibition (Hilioti et al., 2004). In *S. cerevisiae*, Rcn1 expression is induced in a calcineurin-dependent manner, and the phosphorylated protein is itself a substrate for calcineurin (Gorlach et al., 2000; Hilioti and Cunningham, 2003; Hilioti et al., 2004; Kishi et al., 2007). Overexpression of RCAN family members (or their calcineurin binding domain) inhibits calcineurin function in both *S. cerevisiae* and in mammalian cells (Fuentes et al., 2000; Gorlach et al., 2000; Hilioti and Cunningham, 2003; Hilioti et al., 2004; Vega et al., 2002). Thus, RCANs serve as important control elements of the calcineurin cascade that could potentially be manipulated to therapeutically inhibit calcineurin function.

Another key aspect of calcineurin signaling is regulation of cellular calcium homeostasis and signaling. Direct targets of calcineurin include multiple calcium channels (Vcx1, Mid1/Cch1) (Bonilla et al., 2002; Cunningham and Fink, 1996). In *S. cerevisiae*, endoplasmic reticulum stress activates the Mpk1 pathway, which activates a plasma membrane calcium channel composed of Cch1 and Mid1 (Bonilla and Cunningham, 2003; Bonilla et al., 2002). Activation of the channel results in calcium influx and activation of calcineurin, which subsequently feedback inhibits the channel through dephosphorylation (Cunningham and Fink, 1994a). Thus, Mid1 and Cch1 control calcineurin activation. Previous studies in *C. albicans* characterized Mid1 and Cch1 roles in galvano- and thigmo-tropism (Brand et al., 2007). Deletion of either or both calcium channels significantly decreased calcium accumulation. However, the mutants differed in their response to various stimuli: Cch1 appears to play a greater role in hyphal orientation in response to electric fields, while loss of Mid1 had a more significant impact on hyphal tip reorientation in response to physical contract. Interestingly, calcineurin was required for the reorientation of hyphae in an electric field, but not involved in thigmotropism; however, Crz1 was required for both processes (Brand et al., 2007).

In this study, we used mutant analysis to investigate the roles *C. albicans* Rcn1, Mid1, and Cch1 homologs play in the response of *C. albicans* to stress. We found that while each of these proteins is required for the maximal resistance to some stressors, none of them is as important as calcineurin itself. Therefore, each of these proteins likely functions in only part of the calcineurin-signaling pathway.

Materials and methods

Strains and Media

All strains were routinely propagated on YPD medium (1% yeast extract, 2% bacto peptone, 2% dextrose, and 2% bacto agar (DIFCO)). YPD + 300 mM $CaCl_2$ medium was made similarly to YPD except that the media was adjusted to pH 5 prior to autoclaving. The $CaCl_2$ solution

was sterilized separately and the two solutions were mixed after autoclaving. All strains used in this study are listed in Table 1.

Gene Disruptions

All deletion strains were generated in the SC5314 background. All primers used in strain construction are listed in Table 2. For disruption of the *RCN1* gene, two ~500 bp regions with homology to the 5' promoter and 3' terminator region of *RCN1* were PCR amplified, and cloned into plasmid pSFS2A (Reuss et al., 2004) with KpnI/XhoI, and NotI/SacI, respectively, generating plasmid pJLR1. Plasmid pJLR1 was digested with KpnI/SacI and the disruption cassette consisting of the *SAT1* flipper cassette surrounded by ~500 bp of homology flanking *RCN1* was gel purified. SC5314 was transformed with approximately 1 μg of DNA as previously described (Reuss et al., 2004). For all deletion strains, at least 3 independent transformations were performed at each step of disruption and an independent transformant was selected from each transformation for further analysis. Nourseothricin resistant isolates were selected on YPD + 200 μg/ml NAT (Werner). Correct integrants (*RCN1/rcn1::SAT1*) were confirmed by colony PCR and then by Southern blot. At least 3 independent strains that had correctly integrated the disruption cassette were grown overnight in YPM (1% yeast extract, 2% bacto peptone, 2% maltose, and 2% Bactoagar (Difco)) at 30°C and then plated onto YPD + 25 μg/ml NAT. Small colonies which represented those that had excised the *SAT1* cassette (*RCN1/rcn1::frt*) were selected and confirmed by Southern blot. Three independent transformants were selected to undergo a second round of transformation to disrupt the remaining *RCN1* allele.

The *RCN1* complementation cassette to reintroduce the *RCN1* gene at the native locus was generated by PCR amplification of the full-length *RCN1* gene plus ~500 bp of promoter sequence and ~300 bp of terminator, cloned into plasmid pJLR-RCN3 (containing ~500 bp of homology to the 3' terminator of *RCN1* cloned into the NotI/SacI site of pSFS2A) and sequencing revealed no extraneous mutations were found. Because the two alleles of *RCN1* differ in *C. albicans*, plasmids containing each allele were selected (pJLR35 and pJLR37) and used for complementations. Similarly to above, a KpnI/SacI gel purified fragment was used for transformation.

The *rcn1/rcn1 crz1/crz1* strains were isolated by disrupting the *CRZ1* gene in strains JLR36.3, JLR37.1, and JLR38.1. The *CRZ1::SAT1* disruption cassette was obtained by amplifying the *SAT1* flipper cassette using long primers with ~90 bp of homology to the region flanking *CRZ1*. PCRs were carried out using the following conditions: 94°C for 3 minutes, followed by 30 cycles of 94°C for 30 sec, 55°C for 30 sec, 68°C for 4 minutes, and a final extension of 68° C for 15 min. All PCRs were performed using exTAQ polymerase (Takara), and 5% DMSO. An ~4.2 kb PCR fragment was gel purified and 1 μg was used for transformation as discussed above.

The *CCH1* disruption cassette was generated by PCR amplifying two ~500 bp fragments consisting of 5' promoter and 3' terminator regions of the *CCH1* gene, and cloned into plasmid pSFS2A in two steps with KpnI/XhoI, and NotI/SacI respectively, yielding plasmid pJLR3. The procedure followed for disruption was the same as used for *RCN1*. Three independent transformants were confirmed by Southern blot.

The *MID1* disruption cassette was produced by amplifying the *SAT1* flipper cassette using long primers with ~90 bp of homology to the region flanking *MID1* in a similar manner to the disruption of *CRZ1*. Three independent transformants were selected and confirmed by Southern blot. The *MID1* complementation cassette was engineered by PCR amplifying the full *MID1* ORF with ~500 bp of promoter and ~300 bp of terminator. This PCR fragment was cloned into plasmid pJLR-CCH3 (containing ~500 bp 3' *MID1* flanking homology cloned into the NotI/

SacI site of pSFS2A) using KpnI/XhoI. Complementation was carried out as discussed above for *RCN1*. The *mid1/mid1 cch1/cch1* strain was created by disrupting *CCH1* in the *mid1/mid1* strains JLR248, JLR253, and JLR255 using the disruption cassette for *CCH1* described above.

In vitro stress testing

The minimum inhibitory concentrations 80 (MIC_{80}) for all strains was determined using E-test strips (AB Biodisk). For the serial dilution spot assays, strains were grown overnight in YPD at 30°C, washed twice with PBS, and normalized to 1×10^7 cells/ml. 1:10 serial dilutions were spotted onto YPD, YPD containing fluconazole (Diflucan®, Pfizer), FK506 (Prograf, Astellas Pharma US, Inc) or cations. Cells were grown at 30°C and monitored for growth at 24 and 48 hours.

For liquid serum assays, strains were grown overnight in YPD at 30°C, washed twice with PBS, and inoculated into 100% FBS with or without 1 μg FK506 at 2000 cells/ml. Cultures were incubated at 30°C for 24 hours. This temperature was used because it does not affect the interaction between serum and FK506 (calcineurin mutants lose viability at both 30°C and 37°C), and cells incubated at 30°C do not form hyphae, so that the changes in fold population could be accurately measured. Appropriate serial dilutions of the cultures were plated onto YPD for CFU (colony forming unit) counts at 0 and 24 hours. The fold population change was determined by dividing the CFU's at 24 hours by the CFU's at 0 hours.

Heterologous complementation in *S. cerevisiae*

The *C. albicans* and *S. cerevisiae RCN1* homologs were PCR amplified and subcloned into the pCR2.1 TOPO TA vector (Invitrogen) and sequenced. The *RCN1* genes were released by cutting with PvuII and XbaI, gel purified, and cloned into the PvuII/XbaI site of plasmid pYES placing them under the control of the *S. cerevisiae GAL1* promoter. Plasmids were confirmed by digestion and sequencing to have the desired structure. *S. cerevisiae* strains K661 (*vcx1*), K605 (*pmc1*), and K665 (*vcx1 pmc1*) (Cunningham and Fink, 1994b; Cunningham and Fink, 1996; Hilioti and Cunningham, 2003) were transformed with either pYES alone, or plasmids containing either the *C. albicans* or *S. cerevisiae GAL1-RCN1*. Cells were grown to an OD_{600}=0.5 to 0.7, washed with 1 mL 0.1 M LiOAc TE, then resuspended in 0.1 M LiOAc TE. 50 μl of cells were mixed with 2.5 μl salmon sperm DNA (11 mg/ml) (Sigma), 1 μl plasmid, and 300 μl 40% PEG 0.1 M LiOAc The mixture was incubated at 30°C for 30 minutes, and then heat shocked at 42°C for 15 minutes. Cells were then washed with H_2O and plated on SD-ura to select positive transformants. To test for complementation strains carrying pYES alone, *GAL1-ScRCN1*, or *GAL1-CaRCN1* were streaked onto YPD pH 5, YP galactose pH 5, YPD pH 5 with 0.3 M $CaCl_2$, and YP galactose pH 5 with 0.3 M $CaCl_2$. The experiment was performed in duplicate with two different *GAL1-RCN1* plasmids for each species.

Virulence Assays

For the disseminated candidiasis model, 5×10^6 cells of wild-type (SC5314), two independent *rcn1/rcn1* (JLR36.3 and JLR37.1), and two *rcn1/rcn1 + RCN1* strains (JLR180 and JLR323) were injected into the lateral tail vein. Five outbred ICR mice (NCI) were infected per group and monitored for survival. Log rank statistical analysis of the survival data was performed using the PRISM 4.02 program (GraphPad Software, San Diego, Calif.)

The oropharyngeal candidiasis model was performed using mice that were immunosuppressed with cortisone acetate and then orally inoculated as previously described (Park et al., 2005). Seven mice were infected per group. Both histology and colony forming units (CFUs) per gram of oral tissue after 5 days of infection were used to evaluate virulence. Differences in oral

fungal burden among mice infected with the different strains were analyzed using the Wilcox Rank Sum test.

Results

Identification of the *C. albicans RCN1* homolog

The RCAN (Regulator of Calcineurin) family of proteins are calcineurin binding proteins that modulate calcineurin activity in *S. cerevisiae (RCN1), C. neoformans* (*CBP1*), and mammalian cells (*DSCR1/MCIP1*) (Davies et al., 2007; Gorlach et al., 2000; Hilioti and Cunningham, 2003; Strippoli et al., 2000a; Strippoli et al., 2000b). In *S. cerevisiae*, Rcn1 was identified in a screen for proteins that inhibited calcineurin function when overexpressed (Hilioti and Cunningham, 2003). Subsequent studies have shown that the transcription of *RCN1* in *S. cerevisiae* is induced by calcineurin and that Rcn1 has both positive and negative effects on calcineurin function depending on the cellular concentration and phosphorylation state of the protein (Hilioti et al., 2004; Kishi et al., 2007). The *C. albicans* Rcn1 homolog was identified based on the conserved KxFLSPPxSPP domain (Figure 1) (Hilioti and Cunningham, 2003). With the exception of this conserved domain the RCAN family members generally share little homology. Thus, it was expected that this would be the only conserved region of the *C. albicans* Rcn1 protein. The two alleles of *RCN1* in *C. albicans* wild-type strain SC5314 were found to differ by ten amino acids, including 3 amino acid changes and an insert of 7 amino acids in Rcn1-1.

Three independent *C. albicans rcn1/rcn1* disruption mutants were isolated using the *SAT1* flipper cassette method described previously. For complementation experiments, a wild-type allele of *RCN1* was reintroduced at the native locus in all three independent mutants. Two of the mutants were complemented with the *RCN1-2* allele and one was complemented with the *RCN1-1* allele. Both *RCN1* alleles were capable of restoring wild-type phenotypes, and there was no observed functional difference between the two alleles.

rcn1/rcn1 strains are sensitive to LiCl, SDS, and fluconazole

Calcineurin mutants are exquisitely sensitive to cellular stress imposed by cell wall and cell membrane perturbing compounds, as well as various cations (Ca^{2+}, Li^{+}, and Na^{+}) (Bader et al., 2003; Blankenship et al., 2003b; Sanglard et al., 2003). The susceptibility of calcineurin mutants is of particular interest in the clinical setting because calcineurin mutant strains, or wild-type strains in the presence of calcineurin inhibitory drugs (FK506 or Cyclosporin A), are killed by the normally fungistatic azole antifungal drugs (Cruz et al., 2002; Onyewu et al., 2003). Thus, we were interested in whether *rcn1/rcn1* mutants would have similar sensitivities as calcineurin mutant strains. *C. albicans rcn1/rcn1* deletion strains were more susceptible to LiCl, SDS, and fluconazole than the wild-type strain (SC5314), but less sensitive than *cnb1/cnb1* or *cna1/cna1* calcineurin mutant strains (Figure 2). In contrast to calcineurin mutant strains, *rcn1/rcn1* mutants are not hypersensitive to calcium or serum. This suggests that the *RCN1* gene may be involved in the execution of some, but not all calcineurin functions. Similar results have been found in studies of *RCN1* homologs in both *S. cerevisiae* and *C. neoformans. rcn1* deletion in *S. cerevisiae* resulted in a modest sensitivity to Li^{+} that was less severe than a calcineurin mutant phenotype (Gorlach et al., 2000; Hilioti and Cunningham, 2003). Likewise, *C. neoformans cbp1* mutants were still able to grow at 37°C whereas calcineurin mutants are inviable at this temperature. However, deletion of *cbp1* resulted in reduced virulence in a murine model of cryptococcal infection, albeit to a lesser degree than calcineurin mutants (Gorlach et al., 2000).

Neither *rcn1/rcn1* nor *cnb1/cnb1* strains have a virulence defect in an oropharyngeal candidiasis model

Calcineurin mutant strains of *C. albicans* have attenuated virulence in murine models of disseminated disease (Bader et al., 2003; Blankenship et al., 2003b). This attenuation is due to the inability of *C. albicans* calcineurin mutants to withstand the calcium stress imposed by serum, and survive transit through the bloodstream to disseminate throughout the body (Blankenship and Heitman, 2005). Therefore, we tested the virulence of the *rcn1/rcn1* mutant strains in this model. We found that the survival of mice infected with the *rcn1/rcn1* mutant strains was similar to that of mice infected with wild-type *C. albicans* (Figure 3). Therefore, Rcn1 is not essential for maximal virulence during disseminated candidiasis.

Interestingly, further studies have demonstrated that the virulence defect of calcineurin mutant strains is host niche-specific. In contrast to disseminated models of candidiasis, calcineurin mutants exhibit wild-type virulence in both pulmonary and vaginal models of infection (Bader et al., 2006). However, a murine keratitis model demonstrated faster resolution of infection in animals treated with the *cnb1/cnb1* mutant strain or in those animals treated with both a calcineurin inhibitor and antifungal compared with either drug alone (Onyewu et al., 2006). Because oropharyngeal candidiasis is a major manifestation of *Candida* infection, particularly in HIV/AIDS patients (Fidel, 2006; Sweet, 1997), we tested the virulence of both *cnb1/cnb1* and *rcn1/rcn1* strains in this host-niche. After 5 days of infection, the oral fungal burden and histopathology of mice infected with these mutants was similar to mice infected with the wild-type strain (Figure 4 and data not shown). These results, indicate that neither calcineurin nor Rcn1 are required for virulence during oropharyngeal disease (Figure 4).

Overexpression of *C. albicans RCN1* can inhibit *S. cerevisiae* calcineurin function

To determine whether *C. albicans* Rcn1 is capable of interacting with calcineurin, the gene encoding it was expressed heterologously in *S. cerevisiae*. Our rationale for this experiment is as follows. In *S. cerevisiae, RCN1* was originally identified in a screen for genes that, when overexpressed, inhibit calcineurin function and thus promote survival of a *pmc1* strain in the presence of high Ca^{2+} (Hilioti and Cunningham, 2003). In *S. cerevisiae*, calcineurin plays a key role in regulating intracellular compartmentalization of Ca^{2+} (Cunningham and Fink, 1994a). Activated calcineurin induces the expression of the gene encoding Pmc1, a vacuolar Ca^{2+} pump, and thereby promotes influx of calcium into the vacuole. However, calcineurin also simultaneously inhibits the function of another vacuolar calcium pump, Vcx1, through a post-translational mechanism. The activity of either Pmc1 or Vcx1 is required to redistribute intracellular Ca^{2+} and enable the yeast to survive in high calcium conditions. In the presence of a functional calcineurin protein, deletion of *PMC1* is lethal to cells in a high calcium environment; however, if calcineurin function is inhibited then Vcx1 is activated and cells survive calcium stress (Cunningham and Fink, 1994a; Hilioti and Cunningham, 2003). Thus, if overexpression of *RCN1* inhibits calcineurin, *pmc1* strains are rescued in the presence of calcium.

Plasmids were generated allowing expression of both the *S. cerevisiae* and *C. albicans RCN1* genes under the control of the inducible *S. cerevisiae GAL1* promoter. *vcx1*, *pmc1*, or *pmc1 vcx1 S. cerevisiae* strains were transformed with the plasmids. As expected, overexpression of *S. cerevisiae RCN1* rescued *pmc1* strains in the presence of 300 mM calcium (Figure 5). The *C. albicans RCN1* homolog also rescued *pmc1* strains in a high calcium environment, suggesting that *C. albicans RCN1* is capable of interacting with and inhibiting *S. cerevisiae* calcineurin. Due to the highly conserved nature of calcineurin, CaRcn1 is likely to interact similarly with *C. albicans* calcineurin.

rcn1/rcn1 crz1/crz1 double mutants have modestly increased serum, Li^+, and SDS sensitivity compared with either single mutant alone

Thus far, in *C. albicans*, only the Crz1 protein has been shown to act downstream of calcineurin (Karababa et al., 2006; Onyewu et al., 2004; Santos and de Larrinoa, 2005). Similar to *rcn1/rcn1* strains, *crz1/crz1* strains share a subset of cation and drug sensitivities with calcineurin mutant strains, but fail to entirely mimic the sensitivity profile of calcineurin deletion strains. Calcineurin likely has multiple downstream effectors and thus multiple deletion mutations may need to be combined to completely recapitulate calcineurin mutant phenotypes. Thus, we tested whether *rcn1/rcn1 crz1/crz1* strains would be more sensitive to various stressors than either single mutant. Multiple independent double mutant strains were isolated and tested for sensitivity to a variety of stress conditions. The double mutants were found to be more sensitive to Li^+ and SDS than either single mutant (Figure 6). Additionally, the double mutants had significantly reduced growth in serum compared with either single mutant; however, unlike calcineurin mutants, which are killed in serum, the *rcn1/rcn1 crz1/crz1* mutants remained viable (Figure 7). Further experiments will determine whether the calcium stress of serum contributes to the decreased growth of the *rcn1/rcn1 crz1/crz1* strains.

Identification of *C. albicans MID1* and *CCH1* homologs; *mid1/mid1* and *cch1/cch1* strains are sensitive to LiCl and SDS

The *C. albicans MID1* and *CCH1* genes were identified based upon best hit reciprocal BLAST searches using the *S. cerevisiae* homologs. In *S. cerevisiae*, Mid1 and Cch1 form a calcium channel complex that promotes entry of calcium into cells in response to various stresses, including exposure to mating pheromone, endoplasmic reticulum stress, and cations (Bonilla and Cunningham, 2003; Bonilla et al., 2002; Fischer et al., 1997; Iida et al., 1994; Peiter et al., 2005). In response to ER stress, the calcium channels are activated by the Mpk1 pathway and are necessary for calcineurin activation and cell survival (Bonilla and Cunningham, 2003). Activated calcineurin dephosphorylates Mid1, resulting in feedback inhibition of the channel and preventing further influx of calcium (Bonilla et al., 2002). Therefore, the phenotypes of *mid1* and *cch1* cells in response to ER stress are similar to those of a calcineurin mutant. Consistent with the hypothesis that Mid1 and Cch1 form a complex, both the single and double mutants of *S. cerevisiae* have the same phenotype; however, in other species, such as *C. neoformans*, some *CCH1*-independent functions have been observed (Liu et al., 2006). Previous analysis in *C. albicans* on the role of calcium signaling in thigmotropism and galvanotropism reported that while Mid1 played a larger role in thigmotropism, Cch1 was more important for galvanotropism, suggesting that the proteins may have some differences in function (Brand et al., 2007). Recent studies examining the genes regulated in a calcineurin- and Crz1-dependent manner found that *CCH1* is upregulated by activation of either calcineurin or Crz1 (Karababa et al., 2006).

Here both the *MID1* and the *CCH1* gene were deleted with the *SAT1* flipper cassette in the wild-type SC5314 background, and *mid1/mid1 cch1/cch1* double mutants were also generated. The susceptibility of *mid1/mid1* and *cch1/cch1* strains was tested in response to cations (Ca^{2+}, Na^+, and Li^+), SDS, and fluconazole (Figure 8). Both single mutants were sensitive to LiCl and SDS, although not to the same extent as calcineurin mutants. Although neither mutant was sensitive to high Ca^{2+}, the *cch1/cch1* deletion strains were sensitive to low calcium environments. The fluconazole sensitivity of both single mutants was tested using E-test strips, and demonstrated modestly increased susceptibility (MIC_{80}= 0.5 – 0.75) compared with wild-type (MIC_{80}= 1.0 – 1.5). In all cases the *mid1/mid1 cch1/cch1* double mutant behaved similarly to the single mutants, suggesting that at least for the phenotypes studied, neither protein has independent functions.

Discussion

The calcineurin signaling pathway is a key mediator of stress responses in *C. albicans* and has clinical potential as a therapeutic target to enhance the efficacy of the current antifungal armamentarium. Here we have identified additional components of the calcineurin signaling pathway using a candidate gene approach based upon knowledge of the pathway in other organisms. Heterologous expression experiments provide evidence that Rcn1 is capable of inhibiting calcineurin function, similar to the *S. cerevisiae* homolog. Similarly to *S. cerevisiae*, an *rcn1/rcn1* strain shares some similar sensitivities with calcineurin deletion strains; however, they exhibit a more intermediate phenotype and do not completely recapitulate the full severity of the calcineurin mutant phenotype. Microarray studies suggest that Crz1 may be solely responsible for the calcineurin-dependent transcriptional response (Karababa et al., 2006); however, calcineurin likely has other downstream effectors that are direct dephosphorylation targets regulated post-transcriptionally. Thus, to completely recapitulate a calcineurin mutant phenotype it is likely that multiple simultaneous gene deletions may be required. The double *crz1/crz1 rcn1/rcn1* mutant had enhanced sensitivity to several stresses, suggesting that although both proteins interact with calcineurin, they may influence different downstream events. Further studies are needed to establish whether Rcn1 directly interacts with calcineurin in *C. albicans* in a manner similar to *S. cerevisiae*. The high degree of homology of calcineurin between these two fungi makes it likely that the relationship between Rcn1 and calcineurin may be similar in both species.

Several lines of evidence suggest that the Ca^{2+} channel subunits encoded by *MID1* and *CCH1* may play a role in calcineurin signaling. Studies in *S. cerevisiae* have shown that in response to endoplasmic reticulum stress the Mid1/Cch1 calcium channel is activated and the resulting influx of calcium activates calcineurin signaling (Bonilla and Cunningham, 2003; Bonilla et al., 2002). Studies on thigmotropism and galvanotropism showed that Mid1 and Cch1 are required for hyphal reorientation (respectively) and that calcineurin was required for the galvanotropic, but not the thigmotropic responses. However, Crz1 was required for both processes, suggesting that Crz1 could have calcineurin-independent functions (Brand et al., 2007). Additionally, microarray studies in *C. albicans* have shown that calcineurin regulates expression of the *CCH1* gene (Karababa et al., 2006). Deletion analysis of *CCH1* and *MID1* suggests that these genes play a role in tolerance to membrane stress and tolerance to fluconazole, although they have an intermediate phenotype compared with calcineurin mutants. Further studies examining the role that these proteins play in activating calcineurin signaling will be required, including studying the activation of Crz1 in these mutants. For the phenotypes analyzed in this study, both single and double mutants had similar phenotypes suggesting that the two genes act as a complex (as in *S. cerevisiae*) or act in a linear pathway and thus there is no additive effect observed in the double mutant.

Interestingly, these studies also extend our understanding of the host-niche specific role of calcineurin in virulence. Previous studies demonstrated that calcineurin is required for virulence in murine models of systemic candidiasis (Bader et al., 2003; Blankenship et al., 2003b) and candidal keratitis (Onyewu et al., 2006), but is dispensable for virulence in murine vaginal and pulmonary infection models (Bader et al., 2006). Our results suggest that calcineurin is also not required for virulence in oropharyngeal infections. Thus, inhibition of calcineurin alone could be utilized as a novel mechanism of antifungal therapy in certain manifestation of candidal disease, such as bloodstream infections, or keratitis; however, in oropharyngeal, pulmonary, or vaginal candidal infections inhibition of calcineurin alone may not be sufficient. Combining calcineurin inhibition with azoles would likely provide enhanced antifungal activity in all host-niches.

The therapeutic potential of calcineurin inhibitors in combination therapy to enhance the efficacy of current antifungals makes furthering our understanding of this signaling pathway of considerable import. Prior to this study only one direct target of calcineurin, Crz1, had been extensively studied in *C. albicans.* The role of Rcn1 is of interest in *C. albicans* as peptides corresponding to the calcineurin binding segment of human RCAN family members have also been show to inhibit calcineurin function (Mulero et al., 2009), and could potentially be used as starting points to develop novel therapeutics.

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We thank Joachim Morschhauser for the generous gift of the pSFS2A *SAT1* flipper cassette for gene disruptions as well as the *C. albicans* calcineurin A deletion and complemented strains, and Kyle Cunningham for the *S. cerevisiae* strains used in the heterologous expression experiments. We also thank Norma Solis and Trang Phan for assistance with the oropharyngeal candidiasis experiments. This work was supported by NIAID R01 grants AI42159 and AI50438, and by NIDCR R01 grant DE017088.

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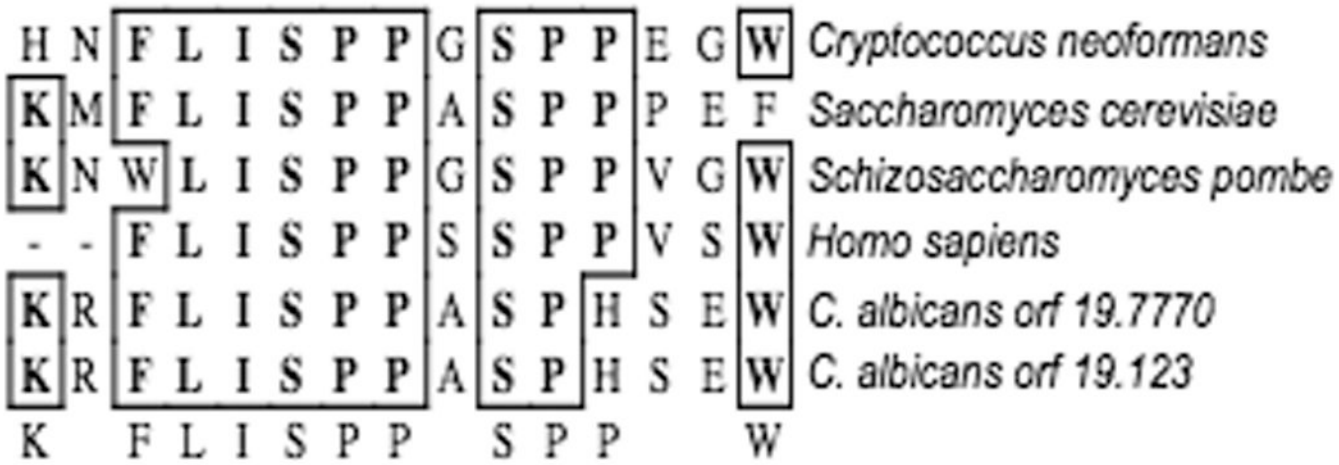

Figure 1. Structure of RCAN family members and *C. albicans* Rcn1

A. Conserved domain of RCAN family members. ClustalW alignment of the conserved domain found in all RCAN family members (created with MacVector™). Shown are *C. neoformans* Cbp1, *S. cerevisiae* Rcn1, *S. pombe* SPAC13G6.15c, *H. sapien* DSCR1, and both *C. albicans* Rcn1 genes.

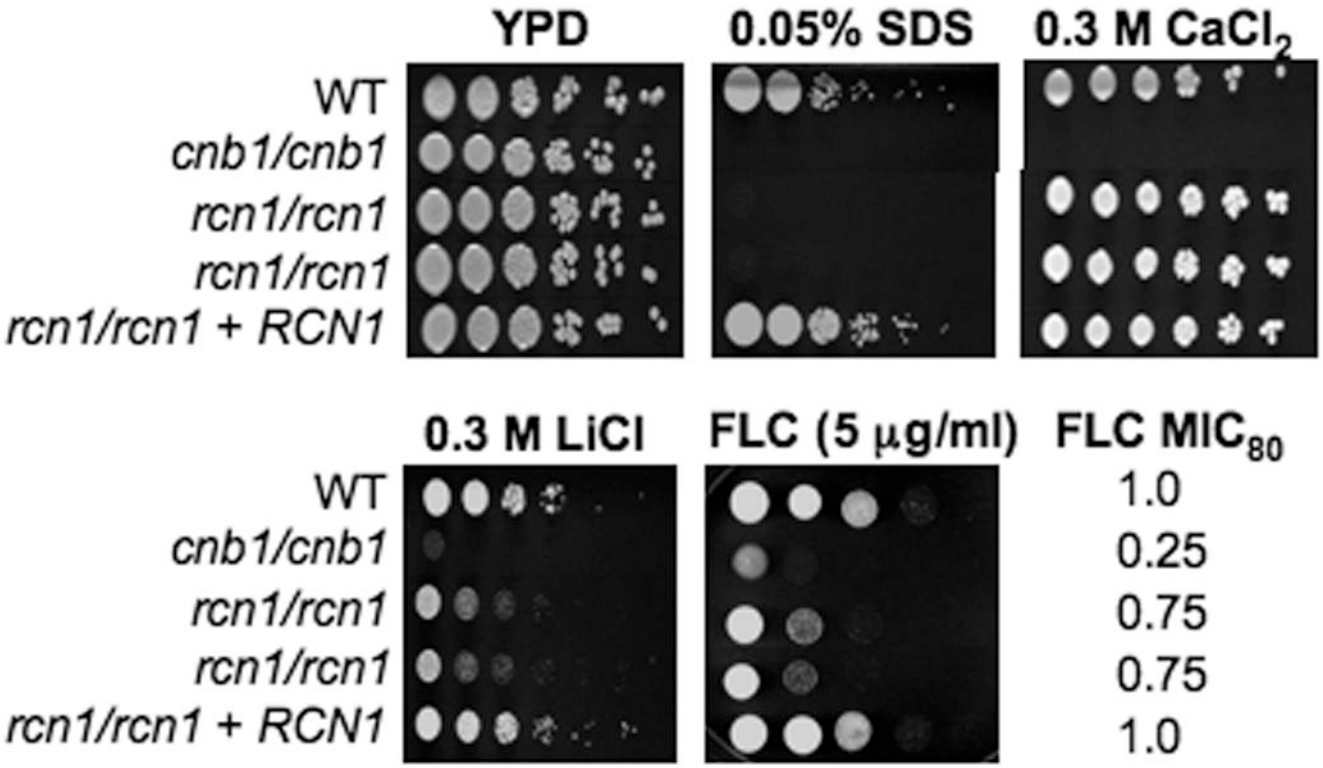

Figure 2. *rcn1/rcn1* strains are sensitive to SDS, Li^+, and fluconazole

Serial dilutions of the wild-type (SC5314), *cnb1/cnb1* (JRB64), *rcn1/rcn1* (JLR36.3 and JLR37.1) and *rcn1/rcn1 +RCN1* (JLR180) strains were spotted onto solid YPD media containing the designated salt or drug concentration and incubated at 30°C for 48 hours. Fluconazole MIC_{80} was determined by E-TEST.

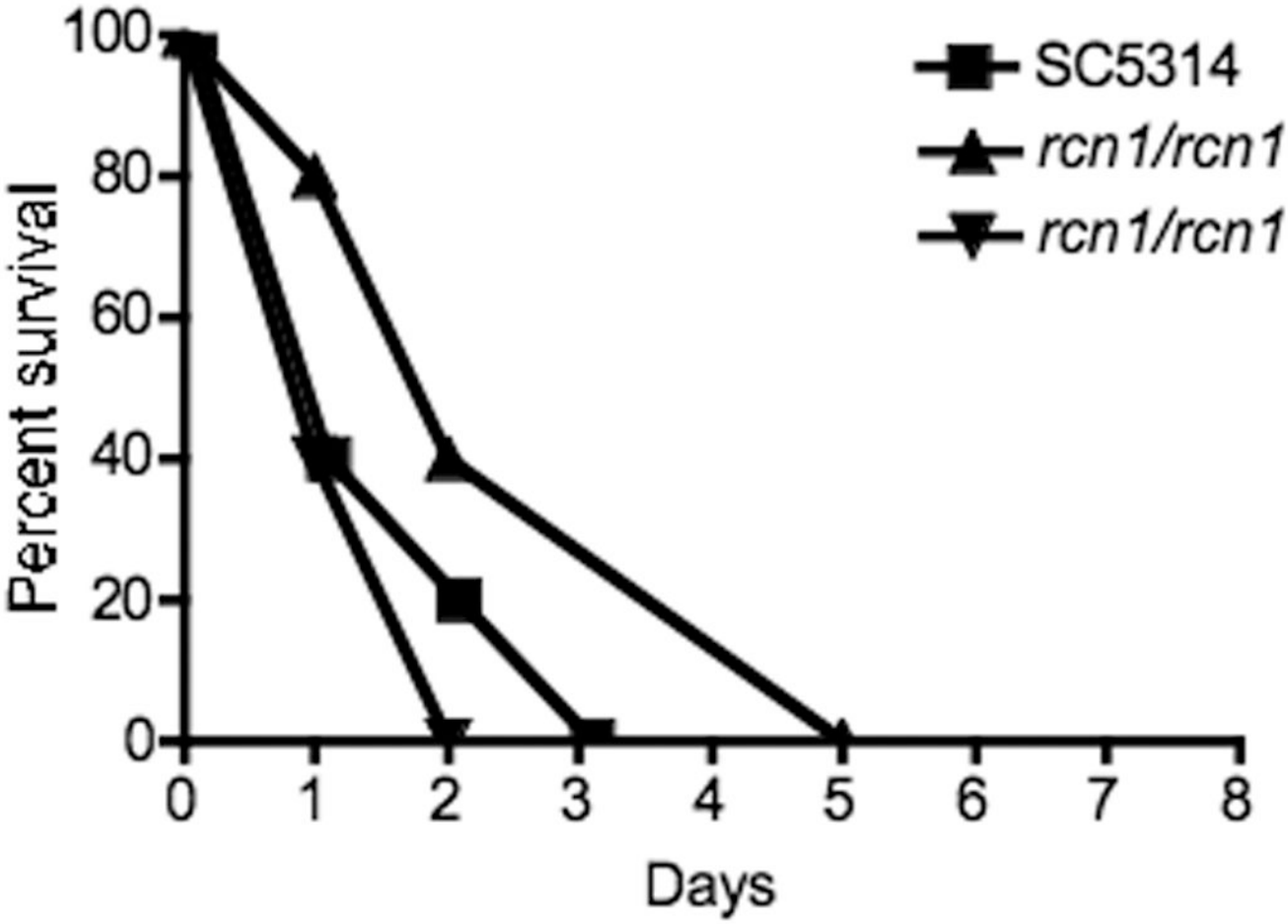

Figure 3. Virulence of *rcn1/rcn1* in a murine disseminated candidiasis model

An inoculum of 5×10^6 cells of wild-type (SC5314), two independent *rcn1/rcn1* (JLR36.3 and JLR37.1), and two *rcn1/rcn1* + *RCN1* (JLR180 and JLR323) strains were injected into the lateral tail vein. Five outbred ICR mice were infected per group. There was no significant difference in the survival between the wild-type and either the mutant or complemented strains ($p = 0.06$). Shown above are the wild-type and deletion mutant strains.

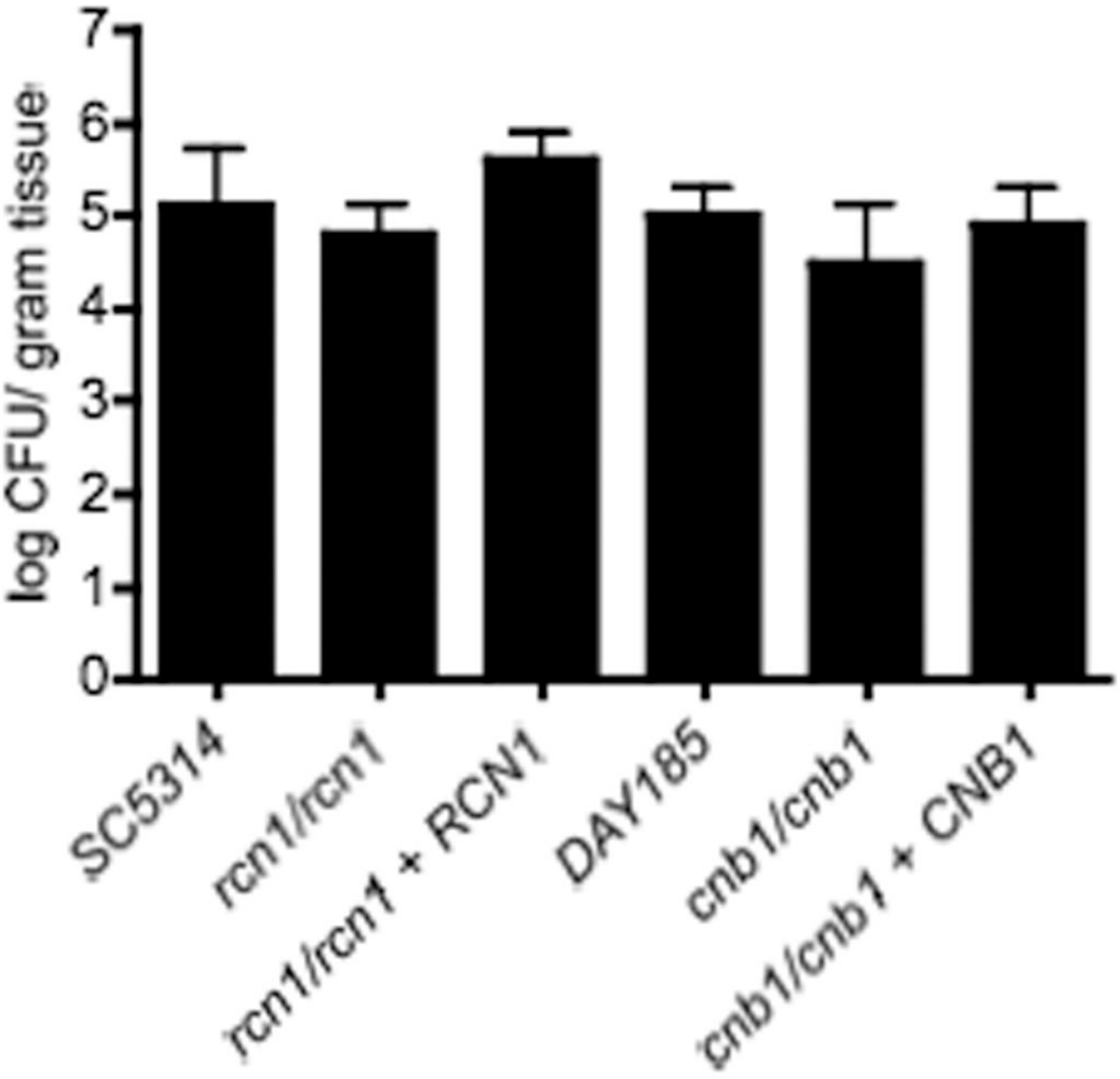

Figure 4. Virulence in a murine oropharyngeal candidiasis model

The oropharyngeal candidiasis infections were performed according to Park, et al. 2005. Seven mice were infected per group. There was no significant difference between the wild-type SC5314 and *rcn1/rcn1* mutant ($p = 0.32$) or between the wild-type DAY185 and *cnb1/cnb1* mutant ($p = 0.21$).

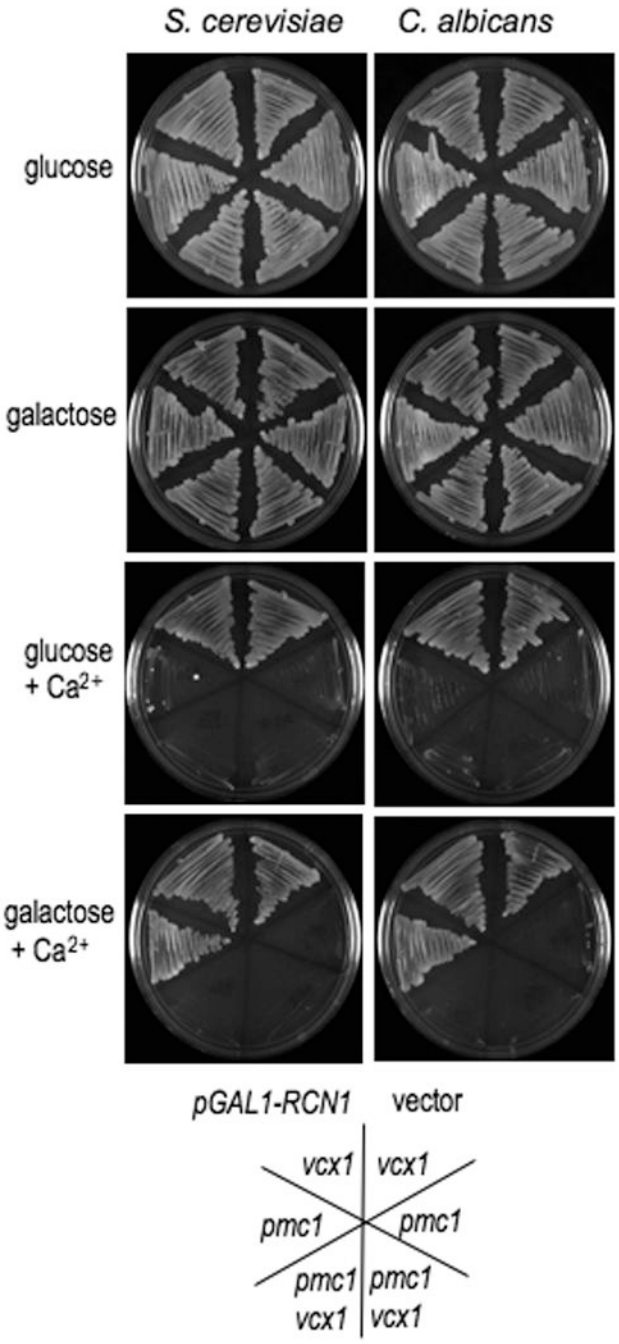

Figure 5. Overexpression of *CaRCN1* rescues the viability of *pmc1* strains

The *C. albicans* or *S. cerevisiae RCN1* gene was cloned into plasmid pYES for expression in *S. cerevisiae* under the control of the *GAL1* promoter. The plasmids were sequence confirmed and then strains *pmc1::TRP1 vcx1 Δ* (K665), *pmc1::TRP1* (K605), and *vcx1* (K661) were transformed. Under conditions of high calcium, calcineurin promotes the expression of *PMC1* and inhibits Vcx1 function. *pmc1* strains are unable to grow at high calcium levels unless calcineurin inhibition of Vcx1 is abolished. Overexpression of either the *CaRCN1* or *ScRCN1* gene allows growth of *pmc1* strains in high calcium suggesting that calcineurin function is inhibited, whereas presence of the vector alone did not effect the growth of *pmc1* strains. Inhibition of calcineurin also prevents the strong upregulation of *PMC1* in response to

high calcium, thus the high calcium conditions were chosen so that the basal expression of *PMC1* was sufficient to support growth when calcineurin was inhibited in the *vcx1* background. Strains were plated on solid YPD medium alone or containing 0.3 M $CaCl_2$. The carbon source was either 2% glucose or 2% galactose, as indicated.

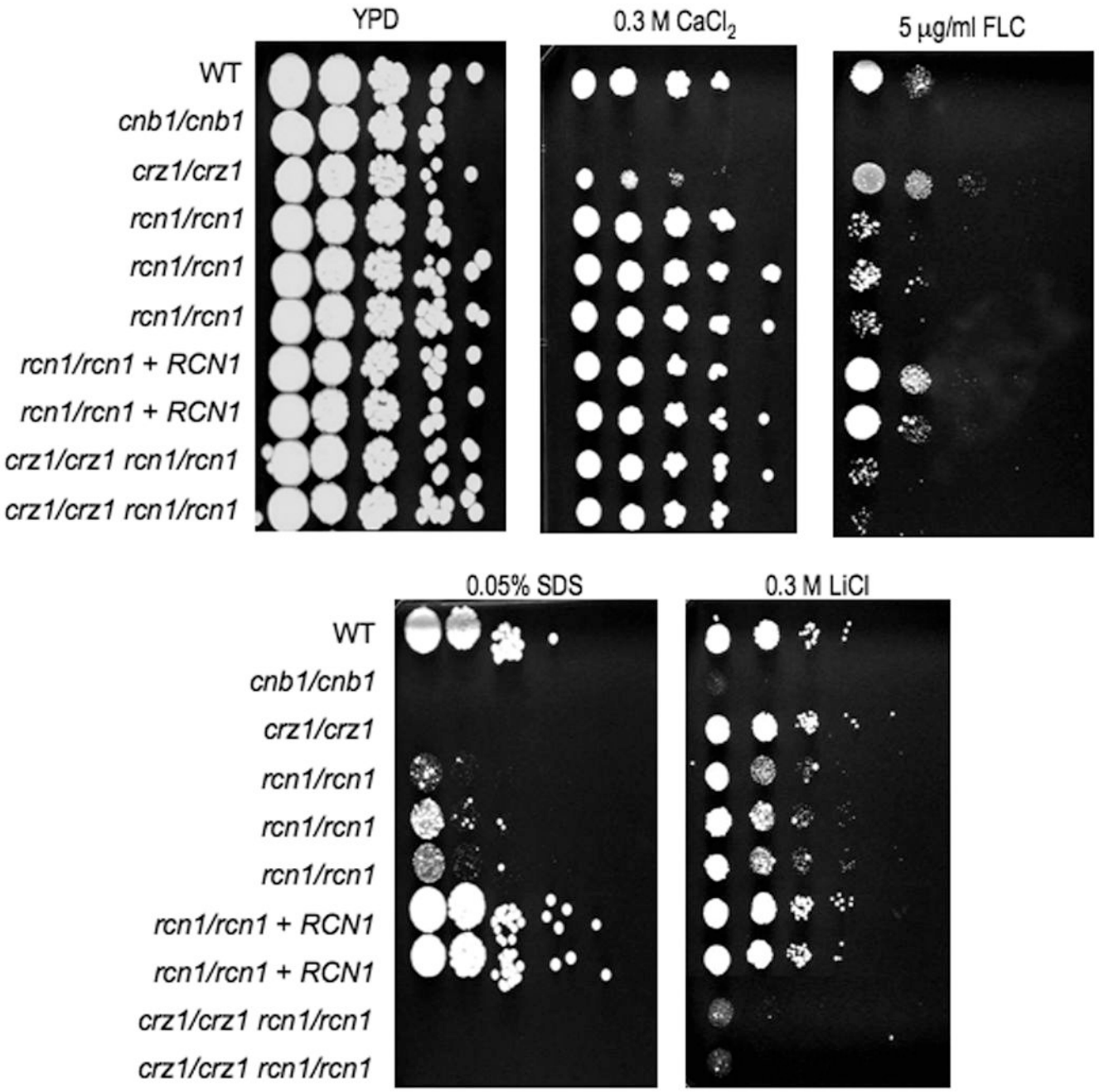

Figure 6. Phenotypic analysis of *rcn1/rcn1 crz1/crz1* strains

Wild-type strain (SC5314), *cnb1/cnb1* (JRB64), *rcn1/rcn1* mutants (JLR36.3, JLR37.1, and JLR38.1), *rcn1/rcn1 +RCN1* complemented strain (JLR180, and JLR323), and *rcn1/rcn1 crz1/crz1* double mutants (JLR548 and JLR578) were serially diluted and spotted onto solid YPD media containing the designated salt or drug concentration, and incubated at 30°C for 48 hours. The double mutants have a more severe phenotype than the single mutants on LiCl and fluconazole (FLU).

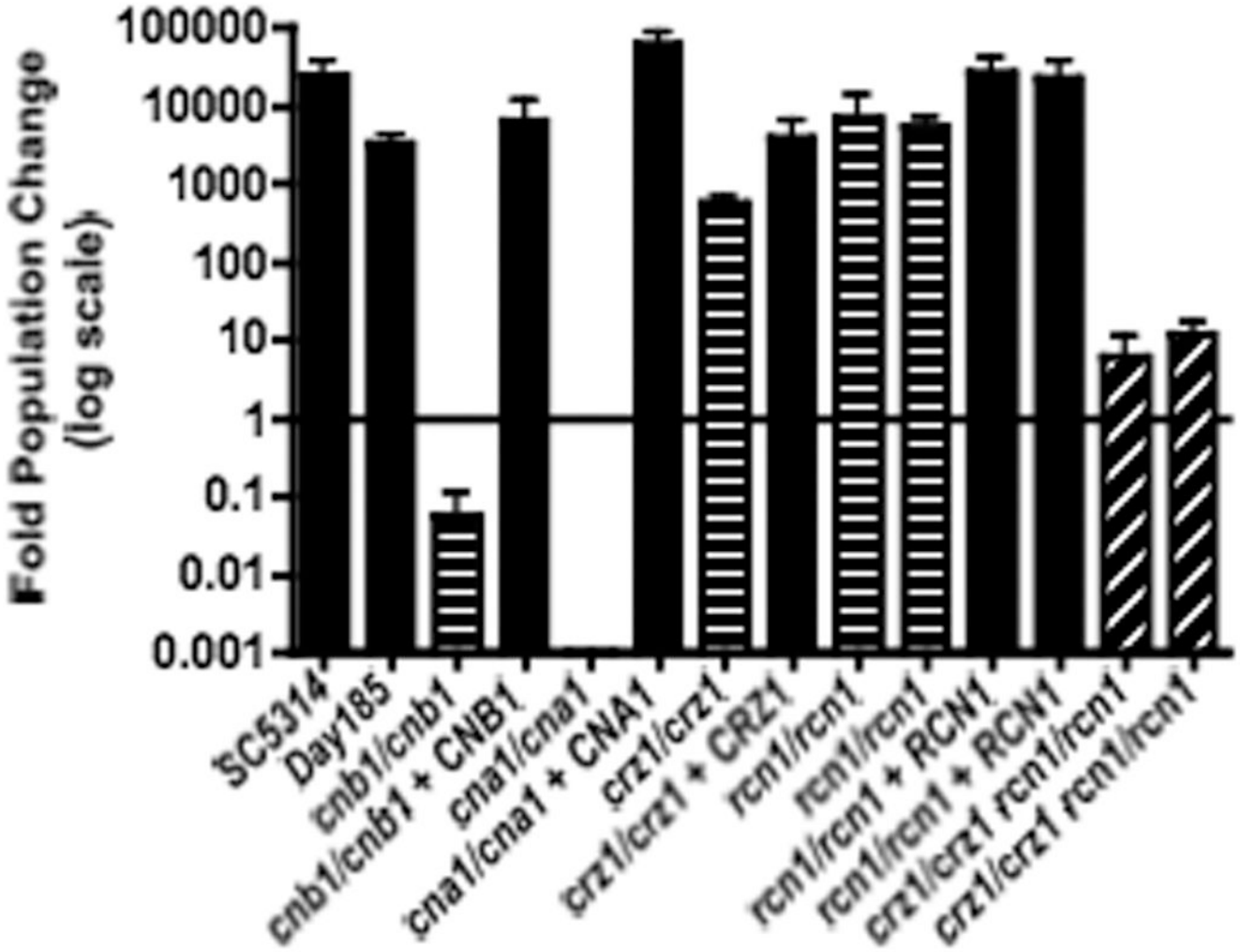

Figure 7. *rcn1/rcn1 crz1/crz1* double mutants have increased serum sensitivity compared to either single mutant alone

Strains were grown for 24 hours in 100% Fetal Bovine Serum at 30°C. Appropriate dilutions from each culture were plated onto YPD at 0 and 24 hours and then incubated at 30°C overnight. Fold population change was determined by dividing CFUs at 24 hours by CFUs at 0 hours. Error bars represent the standard error from two independent experiments. SC5314 and DAY185 served as the wild-type control strains. Strains tested were wild-type (SC5314 and DAY185), *cnb1/cnb1* (JRB64), *cnb1/cnb1 + CNB1* (MCC85), *cna1/cna1* (SCCMP1M4), *cna1/cna1 + CNA1* (SCCMP1M2), *crz1/crz1* (OCC1.1), *crz1/crz1 + CRZ1* (OCC7), *rcn1/rcn1* (JLR36.3 and JLR37.1), *rcn1/rcn1 + RCN1* (JLR180 and JLR323), and *crz1/crz1 rcn1/rcn1* (JLR548 and JLR578)

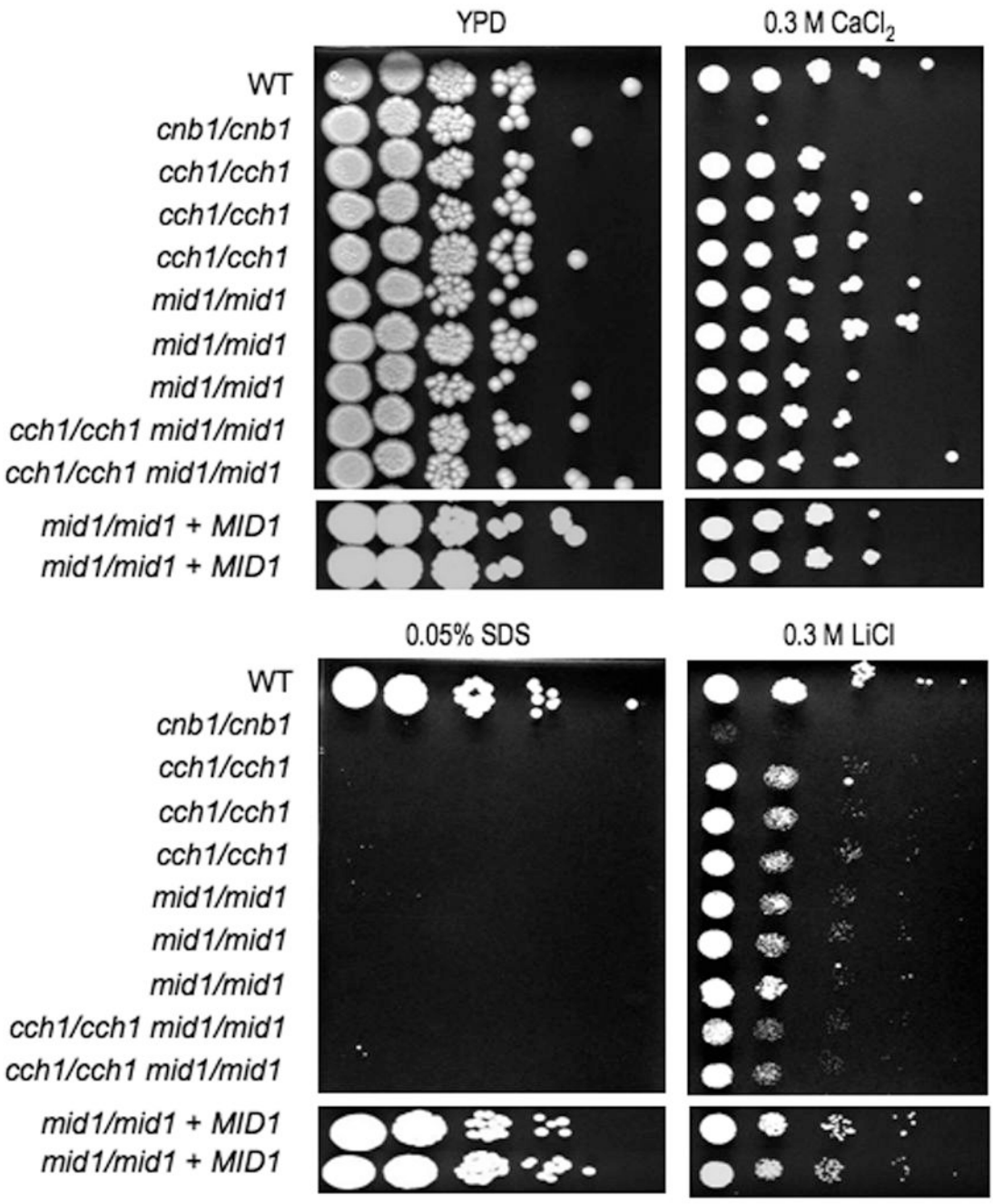

Figure 8. *mid1/mid1* and *cch1/cch1* are sensitive to Li^+ cations and SDS

Serial dilutions of wild-type (SC5314), *mid1/mid1* (JLR248, JLR253, and JLR255), *cch1/cch1* (JLR48, JLR50, and JLR265), *cnb1/cnb1* (JRB64), *mid1/mid1 cch1/cch1* (JLR519 and JLR521), and *mid1/mid1 + MID1* (JLR284 and JLR301) were spotted on solid YPD media containing the designated salt or drug concentration and incubated at 30°C for 48 hours.

Table 1

Strains used in this study.

Strain	Genotype	Background	Source
SC5314			(Gillum et al., 1984)
DAY185	*URA3/ura3Δ::λimm434 HIS1/his1::hisG ARG4/arg4::hisG*	BWP17	(Davis et al., 2000)
JRB64	*ura3Δ::λimm434/ura3Δ::λimm434 his1::hisG::HIS1/his1::hisG arg4::hisG/arg4::hisG cnb1::UAU1/cnb1::URA3*	BWP17	(Blankenship et al., 2003b)
MCC85	*ura3Δ::λimm434/ura3Δ::λimm434 his1::hisG::CNB1-HIS1/his1::hisG arg4::hisG/arg4::hisG cnb1::UAU 1/cnb1::URA3*	BWP17	(Blankenship et al., 2003b)
SCCMP1M4	*cna1::frt/cna1::frt*	SC5314	(Bader, et al., 2006)
SCCMP1M2	*cna1::frt/cna1::frt + CNA1*	SC5314	(Bader, et al., 2006)
OCC1.1	*ura3Δ::λimm434/ura3Δ::λimm434 his1::hisG::HIS1/his1::hisG arg4::hisG/arg4::hisG crz1::UAU1/crz1::URA3*	BWP17	(Onyewu, et al., 2004)
OCC7	*ura3Δ::λimm434/ura3Δ::λimm434 his1::hisG::CRZ1-HIS1/his1::hisG arg4::hisG/arg4::hisG crz1:: UA U1/crz1:: URA3*	BWP17	(Onyewu, et al., 2004)
JLR36.3	*rcn1::frt/rcn1::frt*	SC5314	this study
JLR37.1	*rcn1::frt/rcn1::frt*	SC5314	this study
JLR180	*rcn1::frt/rcn1::frt + RCN1*	JLR36.3	this study
JLR323	*rcn1::frt/rcn1::frt + RCN1*	JLR37.1	this study
JLR248	*mid1::frt/mid1::frt*	SC5314	this study
JLR253	*mid1::frt/mid1::frt*	SC5314	this study
JLR255	*mid1::frt/mid1::frt*	SC5314	this study
JLR284	*mid1::frt/mid1::frt + MID1*	JLR248	this study
JLR301	*mid1::frt/mid1::frt + MID1*	JLR255	this study
JLR48	*cch1::frt/cch1::frt*	SC5314	this study
JLR50	*cch1::frt/cch1::frt*	SC5314	this study
JLR265	*cch1::frt/cch1::frt*	SC5314	this study
JLR548	*crz1/crz1 rcn1/rcn1*	JLR36.3	this study
JLR578	*crz1/crz1 rcn1/rcn1*	JLR37.1	this study
JLR521	*mid1/mid1 cch1/cch1*	JLR248	this study
JLR519	*mid1/mid1 cch1/cch1*	JLR255	this study
K661	*MAT***a** *ade2-1 can1-100 his3-11,15 leu2-3 112 trp1-1 ura3-1 vcx1D*	W303-1A	(Cunningham, et al, 1996)
K605	*MAT***a** *ade2-1 can1-100 his3-11,15 leu2-3 112 trp1-1 ura3-1 pmc1::TRP1*	W303-1A	(Cunningham, et al., 1994)
K665	*MAT***a** *ade2-1 can1-100 his3-11,15 leu2-3 112 trp1-1 ura3-1 pmc1::TRP1 vcx1D*	W303-1A	(Cunningham, et al, 1996)

Table 2

Primers used in this study.

Name	Sequence	Notes
JLR121	AGTCAACGTAGGTACCATGAACCTTAGATAATTACG	*RCN1 5′* flank KpnI
JLR120	TCAACTTCTCGAGCATCAATATTGGTAATGATTAATG	*RCN1 5′* flank XhoI
JLR118	TCAATCATTGCGGCCGCAAGATCATCGATGTAATATGC	*RCN1 3′* flank NotI
JLR119	TACTTACAAGAGCTCGTCTTGGATAGAACATAAAATAC	*RCN1 3′* flank SacI
JLR130	TCAACTTCTCGAGGTCTTGGATAGAACATAAAATAC	*RCN1* complementation 3' XhoI
JLR126	AGTCAACGTAGGTACCCATACACTTGCTTCGCCTCTA	*CCH1 5′* flank KpnI
JLR127	TCAACTTCTCGAGGGTATTGCTGTTATCCATG	*CCH1 5′* flank XhoI
JLR125	TCAATCATTGCGGCCGCGAACTGTCATCTTGGAAAAATGC	*CCH1 3′* flank NotI
JLR124	TACTTACAAGAGCTCCTGATCCAGAAGATTTAGCT	*CCH1 3′* flank SacI
JLR280	ATGTGCTATTCATTAACCACTAGCCTTCATCATGATACCACCTTTT ATTCTACTACTATTTCTAATAATGAACTCATCATGGCCCCCCCTC GAGGAAGTT	*MID1 5′* deletion primer
JLR281	AGAACATAAACAATCAATCAATCGATTCTTCTTCTTCTTTTCTATC TATTATATTCTATTTAAATGAACTATATGACAACGCTCTAGAACT AGTGGATCT	*MID1 3′ deletion primer*
JLR163	TCAACTTCTCGAGGAAGATTTGTCGAGTACTAG	*MID1* complementation 3' XhoI
JLR133	TCAATCATTGCGGCCGCCCTTTACTTTAATGGTTGTCA	*MID1 3′* flank NotI
JLR132	TACTTACAAGAGCTCGAGCTACATTAGTCATAGCAT	*MID1 3′* flank SacI
JLR29	ATTTCCCCTAACAGCATCTTTCCAAGTTCAAATATTTTCCCCTTTT TATATCTAAATTTCATAAATCCCAATCATGTCTGGCCCCCCCTCG AGGAAGTT	*CRZ1 5′* deletion primer
JLR295	TAGAATAAAAAAACAACCAACCAACCAACCAACAGGAATAACT ATCGTGAATGACAACAACCTCAAAAAAAAACTAAGTGCTCTAGA ACTAGTGGATCT	*CRZ1 3′ deletion primer*
JLR470	TCAGGTACTAAGCTGATGCCAAGGAAACCAACAAAT	*Ca RCN1* overexpression 5' PvuII
JLR473	ACGTACTAGATCTAGAGTATGTTGACTTGGATAGAAC	*Ca RCN1* overexpression 3' XbaI
JLR463	TCAGGTACTACAGCTGATGGGTAATATTATAACGGAT	*Sc RCN1* overexpression 5' PvuII
JLR466	ACGTACTAGATCTAGAAAACAATTAACGAGCCATGG	*Sc RCN1* overexpression 3' XbaI