

**Copyright © American Society for Microbiology,
Antimicrobial Agents and Chemotherapy, 54(4), pp.1639-1643; 2010**

Roles of calcineurin and Crz1 in antifungal susceptibility and virulence of *Candida glabrata*

Taiga Miyazaki^{1*}, Shunsuke Yamauchi¹, Tatsuo Inamine², Yosuke Nagayoshi¹, Tomomi Saijo¹, Koichi Izumikawa¹, Masafumi Seki¹, Hiroshi Kakeya¹, Yoshihiro Yamamoto¹, Katsunori Yanagihara¹, Yoshitsugu Miyazaki³ and Shigeru Kohno¹

¹Department of Molecular Microbiology and Immunology, Nagasaki University School of Medicine, 1-7-1 Sakamoto, Nagasaki 852-8501, Japan.

²Department of Pharmacotherapeutics, Nagasaki University Graduate School of Biomedical Sciences, 1-14 Bunkyo-machi, Nagasaki 852-8521, Japan.

³Department of Bioactive Molecules, National Institutes of Infectious Diseases 1-23-1 Toyama, Shinjuku-ku, Tokyo 162-8640, Japan.

*Corresponding author. Mailing address: Department of Molecular Microbiology and Immunology, Nagasaki University School of Medicine, 1-7-1 Sakamoto, Nagasaki 852-8501, Japan. Phone: 81-95-819-7273. Fax: 81-95-849-7285. E-mail: taiga-m@nagasaki-u.ac.jp.

Running title: Role of the calcineurin pathway in *C. glabrata*

ABSTRACT

A *Candida glabrata* calcineurin mutant exhibited increased susceptibility to both azole antifungal and cell-wall damaging agents, and was also attenuated in virulence. Although a mutant lacking the downstream transcription factor Crz1 displayed a cell wall-associated phenotype intermediate to that of the calcineurin mutant and was modestly attenuated in virulence, it did not show increased azole susceptibility. These results suggest that calcineurin regulates both Crz1-dependent and -independent pathways depending on the type of stress.

Infections caused by the opportunistic fungal pathogen *Candida glabrata* are often difficult to treat due in part to its intrinsic or rapidly acquired resistance to azole antifungals (25). Calcineurin, a serine-threonine-specific protein phosphatase (1), has attracted attention as a new target of antifungal therapy based on the studies in several pathogenic fungi including *Candida albicans*, *Cryptococcus neoformans* and *Aspergillus fumigatus* (reviewed in reference 31). To date, very little is known about the calcineurin pathway in *C. glabrata*, although it has been reported that azole antifungals and calcineurin inhibitors have mild synergistic effects against *C. glabrata* wild-type strains (8, 15, 22). The transcription factor Crz1 is a downstream effector of calcineurin and is involved in azole tolerance in *C. albicans* (14, 23, 28); however, a Crz1 homolog in *C. glabrata* has yet to be characterized. Therefore, our objective was to evaluate the potential roles of calcineurin and its downstream target Crz1 in antifungal tolerance and virulence of *C. glabrata* through the characterization of mutant phenotypes.

Calcineurin is a heterodimer consisting of a catalytic A subunit and a Ca²⁺-binding regulatory B subunit, and the association between the two subunits is necessary for phosphatase activity (19). To genetically disrupt calcineurin, we completely deleted the *CNBI* open reading frame (ORF) encoding the regulatory B subunit. *C. glabrata* orthologs of *CNBI* and *CRZI* were identified in the genome database Genolevures (<http://www.genolevures.org/>). The primers and strains used in this study are listed in Tables 1 and 2, respectively. *C. glabrata* cells were propagated in minimal medium (0.7% yeast nitrogen base without amino acids, 2% dextrose) at 30°C, unless otherwise noted. Gene deletion was performed using the one-step PCR-based technique as described previously (13). Briefly, a 1-kb *XhoI* fragment containing *C. glabrata HIS3* was excised from pCgACH (17) and inserted into pBluescript II

SK+ (Stratagene, La Jolla, CA) to yield pBSK-HIS. A deletion construct was amplified from pBSK-HIS with primers tagged with the 100-bp sequences homologous to the flanking regions of the target ORF. Transformation of *C. glabrata* was performed using the lithium acetate (LiAc) protocol (6). Both PCR and Southern blotting were performed to verify that the desired homologous recombination occurred at the target locus without ectopic integration. To construct a centromere-based plasmid containing a *C. glabrata TRP1* marker, a 1,025-bp *SacI-KpnI* fragment containing the *Saccharomyces cerevisiae PGK1* promoter, a polylinker, and the *C. glabrata HIS3* 3' flanking region was excised from pGRB2.2 (12) and inserted into the corresponding site of pCgACT (17) to yield pCgACT-P. The entire ORFs of *C. glabrata CNB1* and *CRZ1* were amplified from the genomic DNA of CBS138 (10) and inserted into pCgACT-P to generate pCgACT-PNB and pCgACT-PRZ, respectively. The constructed plasmids were verified by sequencing before use. Complemented strains were made by transforming mutant strains with a plasmid construct containing the corresponding wild-type gene.

To examine the susceptibility of the generated mutants to antifungal agents, MIC assays were performed with a commercially prepared colorimetric microdilution panel (ASTY; Kyokuto Pharmaceutical Industrial Co., Ltd.) (Table 3) (24). Although increased azole susceptibility was observed in the $\Delta cnb1$ strain, the $\Delta crz1$ strain displayed susceptibility levels similar to, or in some instances lower than, those of wild-type cells. The *CNB1*-complemented strain displayed recovered azole tolerance. Neither the $\Delta cnb1$ nor $\Delta crz1$ strain had an effect on amphotericin B susceptibility. Next, we monitored the percent viability of each strain in the presence and absence of fluconazole as described previously (15). Although the antifungal activity of fluconazole is generally fungistatic, the drug was

fungicidal for the $\Delta cnb1$ strain (Fig. 1). In contrast, the deletion of $CRZI$ did not affect the antifungal activity of fluconazole. These results suggest that calcineurin is involved in azole tolerance via a Crz1-independent pathway in *C. glabrata*.

To examine cell wall-associated phenotypes in the $\Delta cnb1$ and $\Delta crz1$ strains, we examined their susceptibilities to different types of cell wall-damaging agents, including micafungin (inhibitor of β 1,3-glucan synthesis), Congo red (inhibitor of chitin and β -glucan fiber formation), and calcofluor white (inhibitor of chitin polymer assembly), using a previously described method (15, 20, 26). Micafungin was kindly provided by Astellas (Tokyo, Japan) and dissolved in distilled water. Decreased micafungin tolerance was observed in the $\Delta cnb1$ and $\Delta crz1$ strains compared to that in the wild-type control and this was reversed in the reconstituted strains (Fig. 2). While the $\Delta cnb1$ strain showed decreased tolerance to both Congo red and calcofluor white, the $\Delta crz1$ strain exhibited only moderately decreased tolerance to Congo red and was unaffected by calcofluor white exposure (Fig. 2B). These results suggest that the calcineurin-Crz1 pathway plays a role in the response to β 1,3-glucan defects and that calcineurin also regulates a Crz1-independent pathway(s) in response to impaired chitin construction in *C. glabrata*.

To date, the involvement of calcineurin and Crz1 in virulence has not been reported in *C. glabrata*. In contrast to the *C. neoformans* calcineurin mutant (21), deletion of either $CNBI$ or $CRZI$ did not affect cell growth at 37°C in *C. glabrata* (data not shown), which is a necessary prerequisite for comparing virulence levels. We therefore performed a virulence assay using a murine model of disseminated candidiasis as described previously (5). Briefly, groups of 10 female, 8-week-old, BALB/c mice (Charles River Laboratories Japan, Inc., Japan) were infected via the lateral tail vein. The mice were euthanized 7 days after injection

to determine the number of organ CFU. In this study, no mice died before euthanasia. Statistical analyses were performed using the Kruskal-Wallis test with Dunn's posttest for multiple comparisons. A *P* value of <0.05 was considered statistically significant. Mice infected with the $\Delta cnb1$ strain showed significantly reduced fungal burden in all examined organs compared to those infected with the wild-type control and *CNB1*-complemented strains (Fig. 3). Decreased numbers of CFU of the $\Delta crz1$ strain were statistically significant in the kidney but not in the liver and spleen. The results from this assay indicate that the loss of calcineurin results in attenuated virulence while a deletion of *CRZ1* causes only a partial reduction.

This is the first report characterizing the phenotypes of *C. glabrata* *CNB1* and *CRZ1* mutants, and it has identified both similarities and differences with findings for other fungi. For example, the observed *C. glabrata* $\Delta cnb1$ strain phenotype, which is characterized by an increased susceptibility to azoles and cell wall-damaging agents as well as decreased virulence, is consistent with previous findings for other pathogenic fungi, such as *C. albicans* (2-4, 27), *C. neoformans* (11, 18, 21), and *A. fumigatus* (9, 30). To date, an ortholog of *Crz1* in *C. neoformans* has not been identified and a mutant phenotype associated with azole susceptibility in *A. fumigatus* has yet to be reported; thus, the full importance of this transcriptional factor is not clear for these fungi. Although the virulence of a $\Delta crz1$ mutant is highly attenuated in *A. fumigatus* (7, 29), this mutation has little effect on virulence in both *C. albicans* (14, 23) and *C. glabrata* (Fig. 3). In contrast to that in *C. albicans* (14, 23, 28), the loss of *Crz1* did not result in increased azole susceptibility in *C. glabrata*. In addition, the *C. glabrata* $\Delta crz1$ strain exhibited increased susceptibility to micafungin and Congo red but not to calcofluor white. Taken together, these results indicate that calcineurin-mediated *Crz1*

regulation is dependent upon the type of stress and that the regulatory mechanisms vary among fungal species. Further characterization of these mutant phenotypes will help to discover a novel and conserved calcineurin target in pathogenic fungi.

Acknowledgments

We thank Hironobu Nakayama for providing *C. glabrata* strains 2001T and 2001HT and plasmids pCgACH and pCgACT and Brendan Cormack for providing pGRB2.2. This research was partially supported by a Grant-in-Aid for Scientific Research (no. 19790324 to T.M. and no. 21390305 to S.K.) from the Japanese Ministry of Education, Culture, Sports, Science and Technology, a grant from the Global Centers of Excellence Programs, Nagasaki University, and grants from the Ministry of Health, Labour and Welfare (H20-nanchi-ippan-035, H20-shinko-ippan-012, and H20-shinko-ippan-015 to Y.M.).

References

1. **Aramburu, J., A. Rao, and C. B. Klee.** 2000. Calcineurin: from structure to function. *Curr Top Cell Regul* **36**:237-95.
2. **Bader, T., B. Bodendorfer, K. Schroppel, and J. Morschhauser.** 2003. Calcineurin is essential for virulence in *Candida albicans*. *Infect Immun* **71**:5344-54.
3. **Bader, T., K. Schroppel, S. Bentink, N. Agabian, G. Kohler, and J. Morschhauser.** 2006. Role of calcineurin in stress resistance, morphogenesis, and virulence of a *Candida albicans* wild-type strain. *Infect Immun* **74**:4366-9.
4. **Blankenship, J. R., F. L. Wormley, M. K. Boyce, W. A. Schell, S. G. Filler, J. R. Perfect, and J. Heitman.** 2003. Calcineurin is essential for *Candida albicans* survival in serum and virulence. *Eukaryot Cell* **2**:422-30.
5. **Chen, K. H., T. Miyazaki, H. F. Tsai, and J. E. Bennett.** 2007. The bZip transcription factor Cgap1p is involved in multidrug resistance and required for activation of multidrug transporter gene CgFLR1 in *Candida glabrata*. *Gene* **386**:63-72.
6. **Cormack, B. P., and S. Falkow.** 1999. Efficient homologous and illegitimate recombination in the opportunistic yeast pathogen *Candida glabrata*. *Genetics* **151**:979-87.
7. **Cramer, R. A., Jr., B. Z. Perfect, N. Pinchai, S. Park, D. S. Perlin, Y. G. Asfaw, J. Heitman, J. R. Perfect, and W. J. Steinbach.** 2008. Calcineurin target CrzA regulates conidial germination, hyphal growth, and pathogenesis of *Aspergillus fumigatus*. *Eukaryot Cell* **7**:1085-97.
8. **Cruz, M. C., A. L. Goldstein, J. R. Blankenship, M. Del Poeta, D. Davis, M. E.**

- Cardenas, J. R. Perfect, J. H. McCusker, and J. Heitman.** 2002. Calcineurin is essential for survival during membrane stress in *Candida albicans*. *Embo J* **21**:546-59.
9. **da Silva Ferreira, M. E., T. Heinekamp, A. Hartl, A. A. Brakhage, C. P. Semighini, S. D. Harris, M. Savoldi, P. F. de Gouvea, M. H. de Souza Goldman, and G. H. Goldman.** 2007. Functional characterization of the *Aspergillus fumigatus* calcineurin. *Fungal Genet Biol* **44**:219-30.
10. **Dujon, B., D. Sherman, G. Fischer, P. Durrens, S. Casaregola, I. Lafontaine, J. De Montigny, C. Marck, C. Neuveglise, E. Talla, N. Goffard, L. Frangeul, M. Aigle, V. Anthouard, A. Babour, V. Barbe, S. Barnay, S. Blanchin, J. M. Beckerich, E. Beyne, C. Bleykasten, A. Boisrame, J. Boyer, L. Cattolico, F. Confanioleri, A. De Daruvar, L. Despons, E. Fabre, C. Fairhead, H. Ferry-Dumazet, A. Groppi, F. Hantraye, C. Hennequin, N. Jauniaux, P. Joyet, R. Kachouri, A. Kerrest, R. Koszul, M. Lemaire, I. Lesur, L. Ma, H. Muller, J. M. Nicaud, M. Nikolski, S. Oztas, O. Ozier-Kalogeropoulos, S. Pellenz, S. Potier, G. F. Richard, M. L. Straub, A. Suleau, D. Swennen, F. Tekaiia, M. Wesolowski-Louvel, E. Westhof, B. Wirth, M. Zeniou-Meyer, I. Zivanovic, M. Bolotin-Fukuhara, A. Thierry, C. Bouchier, B. Caudron, C. Scarpelli, C. Gaillardin, J. Weissenbach, P. Wincker, and J. L. Souciet.** 2004. Genome evolution in yeasts. *Nature* **430**:35-44.
11. **Fox, D. S., M. C. Cruz, R. A. Sia, H. Ke, G. M. Cox, M. E. Cardenas, and J. Heitman.** 2001. Calcineurin regulatory subunit is essential for virulence and mediates interactions with FKBP12-FK506 in *Cryptococcus neoformans*. *Mol Microbiol* **39**:835-49.

12. **Frieman, M. B., J. M. McCaffery, and B. P. Cormack.** 2002. Modular domain structure in the *Candida glabrata* adhesin Epa1p, a beta1,6 glucan-cross-linked cell wall protein. *Mol Microbiol* **46**:479-92.
13. **Gola, S., R. Martin, A. Walther, A. Dunkler, and J. Wendland.** 2003. New modules for PCR-based gene targeting in *Candida albicans*: rapid and efficient gene targeting using 100 bp of flanking homology region. *Yeast* **20**:1339-47.
14. **Karababa, M., E. Valentino, G. Pardini, A. T. Coste, J. Bille, and D. Sanglard.** 2006. CRZ1, a target of the calcineurin pathway in *Candida albicans*. *Mol Microbiol* **59**:1429-51.
15. **Kaur, R., I. Castano, and B. P. Cormack.** 2004. Functional genomic analysis of fluconazole susceptibility in the pathogenic yeast *Candida glabrata*: roles of calcium signaling and mitochondria. *Antimicrob Agents Chemother* **48**:1600-13.
16. **Kitada, K., E. Yamaguchi, and M. Arisawa.** 1995. Cloning of the *Candida glabrata* TRP1 and HIS3 genes, and construction of their disruptant strains by sequential integrative transformation. *Gene* **165**:203-6.
17. **Kitada, K., E. Yamaguchi, and M. Arisawa.** 1996. Isolation of a *Candida glabrata* centromere and its use in construction of plasmid vectors. *Gene* **175**:105-8.
18. **Kraus, P. R., D. S. Fox, G. M. Cox, and J. Heitman.** 2003. The *Cryptococcus neoformans* MAP kinase Mpk1 regulates cell integrity in response to antifungal drugs and loss of calcineurin function. *Mol Microbiol* **48**:1377-87.
19. **Kraus, P. R., and J. Heitman.** 2003. Coping with stress: calmodulin and calcineurin in model and pathogenic fungi. *Biochem Biophys Res Commun* **311**:1151-7.
20. **Miyazaki, T., H. F. Tsai, and J. E. Bennett.** 2006. Kre29p is a novel nuclear protein

- involved in DNA repair and mitotic fidelity in *Candida glabrata*. *Curr Genet* **50**:11-22.
21. **Odom, A., S. Muir, E. Lim, D. L. Toffaletti, J. Perfect, and J. Heitman.** 1997. Calcineurin is required for virulence of *Cryptococcus neoformans*. *Embo J* **16**:2576-89.
 22. **Onyewu, C., J. R. Blankenship, M. Del Poeta, and J. Heitman.** 2003. Ergosterol biosynthesis inhibitors become fungicidal when combined with calcineurin inhibitors against *Candida albicans*, *Candida glabrata*, and *Candida krusei*. *Antimicrob Agents Chemother* **47**:956-64.
 23. **Onyewu, C., F. L. Wormley, Jr., J. R. Perfect, and J. Heitman.** 2004. The calcineurin target, Crz1, functions in azole tolerance but is not required for virulence of *Candida albicans*. *Infect Immun* **72**:7330-3.
 24. **Pfaller, M. A., S. Arikan, M. Lozano-Chiu, Y. Chen, S. Coffman, S. A. Messer, R. Rennie, C. Sand, T. Heffner, J. H. Rex, J. Wang, and N. Yamane.** 1998. Clinical evaluation of the ASTY colorimetric microdilution panel for antifungal susceptibility testing. *J Clin Microbiol* **36**:2609-12.
 25. **Pfaller, M. A., and D. J. Diekema.** 2007. Epidemiology of invasive candidiasis: a persistent public health problem. *Clin Microbiol Rev* **20**:133-63.
 26. **Ram, A. F., and F. M. Klis.** 2006. Identification of fungal cell wall mutants using susceptibility assays based on Calcofluor white and Congo red. *Nat Protoc* **1**:2253-6.
 27. **Sanglard, D., F. Ischer, O. Marchetti, J. Entenza, and J. Bille.** 2003. Calcineurin A of *Candida albicans*: involvement in antifungal tolerance, cell morphogenesis and virulence. *Mol Microbiol* **48**:959-76.
 28. **Santos, M., and I. F. de Larrinoa.** 2005. Functional characterization of the *Candida*

albicans CRZ1 gene encoding a calcineurin-regulated transcription factor. *Curr Genet* **48**:88-100.

29. **Soriani, F. M., I. Malavazi, M. E. da Silva Ferreira, M. Savoldi, M. R. Von Zeska Kress, M. H. de Souza Goldman, O. Loss, E. Bignell, and G. H. Goldman.** 2008. Functional characterization of the *Aspergillus fumigatus* CRZ1 homologue, CrzA. *Mol Microbiol* **67**:1274-91.
30. **Steinbach, W. J., R. A. Cramer, Jr., B. Z. Perfect, Y. G. Asfaw, T. C. Sauer, L. K. Najvar, W. R. Kirkpatrick, T. F. Patterson, D. K. Benjamin, Jr., J. Heitman, and J. R. Perfect.** 2006. Calcineurin controls growth, morphology, and pathogenicity in *Aspergillus fumigatus*. *Eukaryot Cell* **5**:1091-103.
31. **Steinbach, W. J., J. L. Reedy, R. A. Cramer, Jr., J. R. Perfect, and J. Heitman.** 2007. Harnessing calcineurin as a novel anti-infective agent against invasive fungal infections. *Nat Rev Microbiol* **5**:418-30.

TABLE 1. Primers used in this study

Primer ^a	Target gene	Sequence (5' – 3') ^b
For gene deletion		
CgCNB 100-F	<i>CNBI</i>	<i>GTATGTGATGCTTCTCACAGGGTTCAGACGGTTACAT ACCATCGCTTGAGAGTCATAGTAAATGTTCAAGTTC CGATTAAATCATGCTTTCTCTTTGATAATACGACTC ACTATAGGGC</i>
CgCNB 100-R	<i>CNBI</i>	<i>GCGAACTCTGAAATGTAGATCAAGGATTATTCTGTCC TTGAAATGGGTGTTGATGTCCCTCACTAGGAAAGACA ACCACTTTACTATTGTAAGGGGTGACGCTCTAGAA CTAGTGGATCC</i>
CgCRZ 100-F	<i>CRZI</i>	<i>GATAACGAGTTGGACGCCCTCTTTTGGAAAGTCTGTTC TGGTTGCAGATGCTTATAGACCCTGGATCAAGCACTT CATTCATTGGGATTACAGCTTTTCTAATACGACTC ACTATAGGGC</i>
CgCRZ 100-R	<i>CRZI</i>	<i>CACAATCTTGATTCTGAAGAAAAAATTTATCATTAAA AATACTGGAGGTTTGTGTTAATTTATTCCAAAGTAACA CCCATCTCAGTTGCTTGAATATTCGCTCTAGAACTA GTGGATCC</i>
For gene cloning		
CgCNB1-F2-5P	<i>CNBI</i>	ATCAAGGGAAATGGGAGC
CgCNB1-R1-5P	<i>CNBI</i>	CGCCCTAAGTTACATCTCTCCTCG
CgCRZ1-F1-E	<i>CRZI</i>	<u>CGGAATTC</u> ATGGGCGATAACGAAGAGGA
CgCRZ1-R1938-E	<i>CRZI</i>	<u>CGGAATTC</u> TATTCCAAAGTAACACCCATCTCA

^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. Restriction sites are underlined.

TABLE 2. Strains used in this study.

Strain	Genotype or description	Reference or source
CBS138	Wild-type	(10)
2001T	$\Delta trp1$ (a derivative of CBS138)	(16)
2001HT	$\Delta his3, \Delta trp1$ (made from 2001T)	(16)
TG11	2001T containing pCgACT-P	This study
TG161	$\Delta cnb1::HIS3, \Delta trp1$ (made from 2001HT)	This study
TG162	TG161 containing pCgACT-P	This study
TG163	TG161 containing pCgACT-PNB	This study
TG171	$\Delta crz1::HIS3, \Delta trp1$ (made from 2001HT)	This study
TG172	TG171 containing pCgACT-P	This study
TG173	TG171 containing pCgACT-PRZ	This study

TABLE 3. Antifungal susceptibilities of *C. glabrata* strains

Strain (genotype)	MIC ($\mu\text{g/ml}$) ^a				
	FLC	MCZ	ITC	VRC	AMB
TG11 (wild-type)	16	0.5	2	0.25	0.5
TG162 (Δcnb1)	4	0.125	0.5	0.125	0.5
TG163 ($\Delta\text{cnb1} + \text{CNB1}$)	16	0.5	2	0.25	0.5
TG172 (Δcrz1)	32	1	1	0.5	0.5
TG173 ($\Delta\text{crz1} + \text{CRZ1}$)	16	0.5	1	0.25	0.5

^a FLC, fluconazole; MCZ, miconazole; ITC, itraconazole; VRC, voriconazole; AMB, amphotericin B.

Figure legends

Fig. 1. Time-kill curves of *C. glabrata* wild-type and mutant strains exposed to fluconazole. Logarithmic-phase cells (5×10^5 CFU/ml) were incubated in minimal medium with agitation in the presence or absence of fluconazole at the indicated concentrations. The total number of cells was counted using a hemocytometer, and the number of viable cells was determined by plating the appropriate dilutions on yeast extract-peptone-dextrose (YPD) plates. The data are expressed as the percentages of viability and represent the means and standard deviations for three independent experiments.

Fig. 2. Susceptibilities of *C. glabrata* wild-type and mutant strains to cell wall-damaging agents. (A) Logarithmic-phase cells (2.5×10^3 CFU/ml) were incubated in minimal medium in either the presence or absence of micafungin, and the optical density at 600 nm (OD_{600}) was measured after 24 h (left panel). The percentages of absorbance were calculated from the OD_{600} of each culture after 24 h of incubation in the presence of 0.03 $\mu\text{g/ml}$ micafungin relative to those in the absence of micafungin (right panel). Data represent the means and standard deviations for three independent experiments. (B) Serial 10-fold dilutions of *C. glabrata* log-phase cells were spotted onto minimal medium plates containing micafungin, Congo red, or calcofluor white at the indicated concentrations. Plates were incubated at 30°C for 48 h. All sensitivity tests were repeated at least three times. *C. glabrata* strains were as follows: wild-type, 2001T containing an empty vector (strain TG11); $\Delta cnb1$, a $\Delta cnb1$ strain containing an empty vector (strain TG162); $\Delta cnb1 + CNB1$, a $CNB1$ -complemented strain made with pCgACT-PNB (strain TG163); $\Delta crz1$, a

$\Delta crz1$ strain containing an empty vector (strain TG172); and $\Delta crz1 + CRZ1$, a $CRZ1$ -complemented strain made with pCgACT-PRZ (strain TG173).

Fig. 3. Virulence assay using a mouse model of disseminated candidiasis. Groups of 10 mice were intravenously inoculated with 8×10^7 cells for each *C. glabrata* strain. Three target organs (liver, spleen, and bilateral kidneys) were excised 7 days after injection. Appropriate dilutions of organ homogenates were plated, and the numbers of CFU were counted after 3 days of incubation at 30°C. Numbers of recovered CFU from each organ are indicated for individual mice in the scatter plots. The geometric mean is shown as a bar. Representative data of two independent experiments are shown. *C. glabrata* strains are as follows: wild-type, TG11 (wild-type control); $\Delta cnb1$, TG162 ($\Delta cnb1$ strain containing an empty vector); $\Delta cnb1 + CNB1$, TG163 ($CNB1$ -complemented strain made with pCgACT-PNB); $\Delta crz1$, TG172 ($\Delta crz1$ strain containing an empty vector); $\Delta crz1 + CRZ1$, TG173 ($CRZ1$ -complemented strain made with pCgACT-PRZ). ¶ , $P < 0.05$ (Kruskal-Wallis test with Dunn's posttest).

Fig. 1

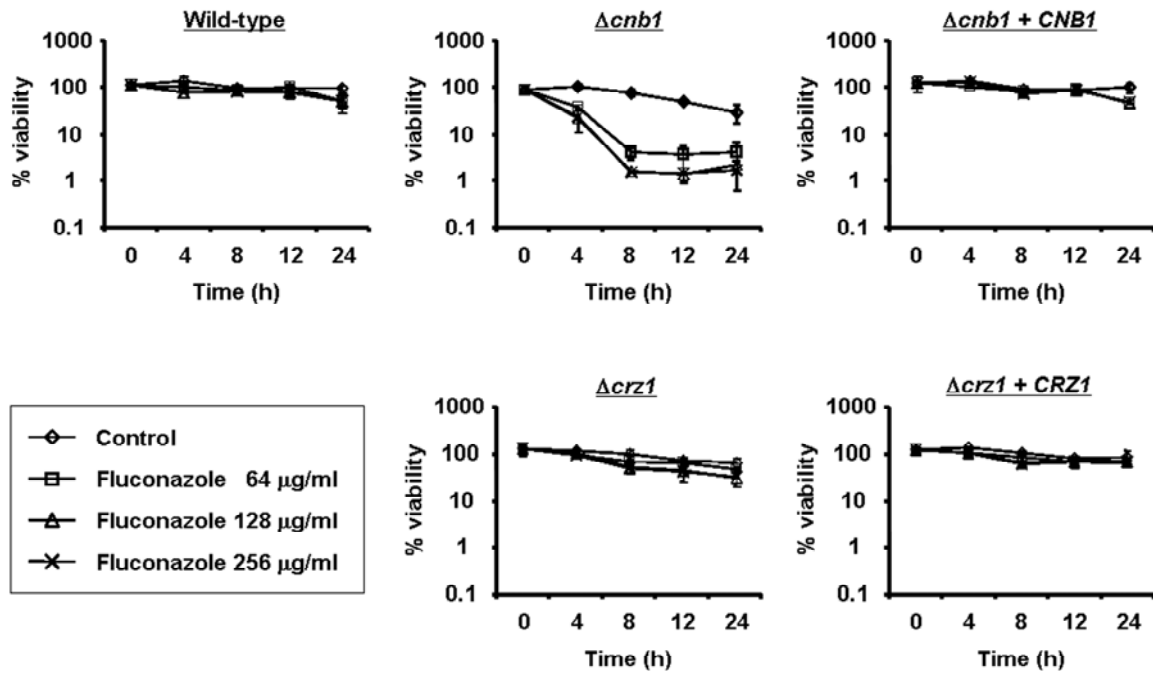


Fig. 2

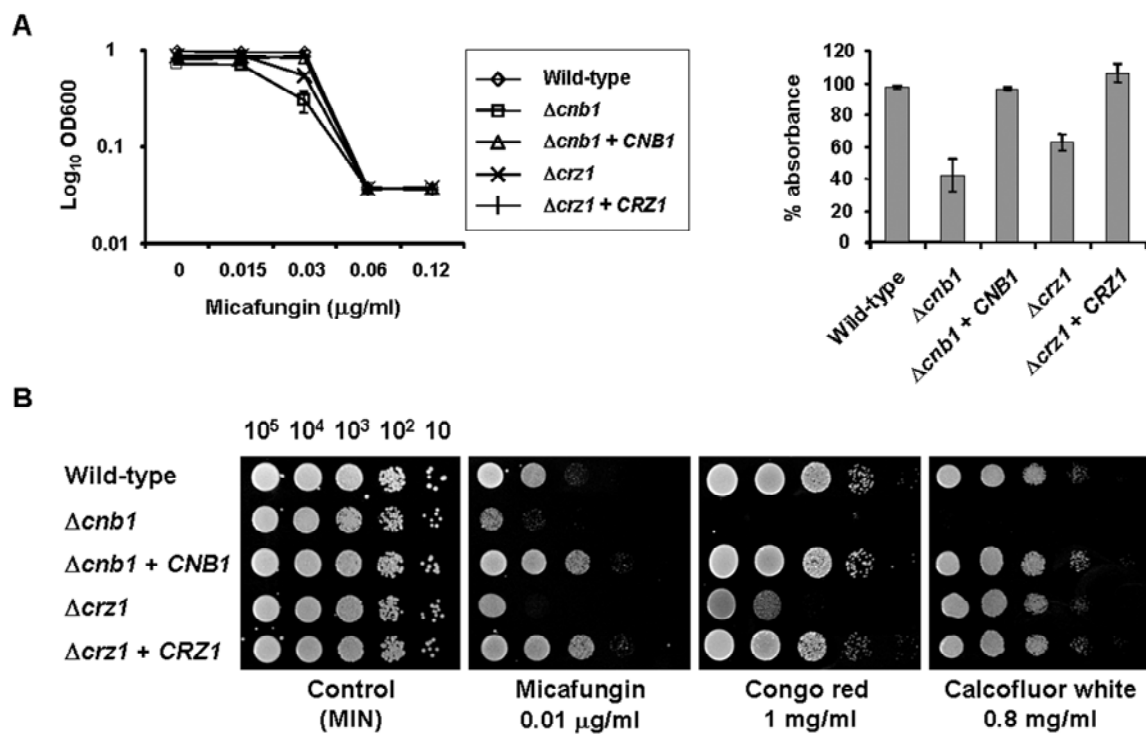


Fig. 3

