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Environmentally contingent control of *Candida albicans* cell wall integrity by transcriptional regulator Cup9

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Abstract

The fungal pathogen *Candida albicans* is surrounded by a cell wall that is the target of caspofungin and other echinocandin antifungals. *Candida albicans* can grow in several morphological forms, notably budding yeast and hyphae. Yeast and hyphal forms differ in cell wall composition, leading us to hypothesize that there may be distinct genes required for yeast and hyphal responses to caspofungin. Mutants in 27 genes reported previously to be caspofungin hypersensitive under yeast growth conditions were all caspofungin hypersensitive under hyphal growth conditions as well. However, a screen of mutants defective in transcription factor genes revealed that Cup9 is required for normal caspofungin tolerance under hyphal and not yeast growth conditions. In a hyphal-defective *efg1*Δ/Δ background, Cup9 is still required for normal caspofungin tolerance. This result argues that Cup9 function is related to growth conditions rather than cell morphology. RNA-seq conducted under hyphal growth conditions indicated that 361 genes were up-regulated and 145 genes were down-regulated in response to caspofungin treatment. Both classes of caspofungin-responsive genes were enriched for cell wall-related proteins, as expected for a response to disruption of cell wall integrity and biosynthesis. The *cup9*Δ/Δ mutant, treated with caspofungin, had reduced RNA levels of 40 caspofungin up-regulated genes, and had increased RNA levels of 8 caspofungin down-regulated genes, an indication that Cup9 has a narrow rather than global role in the cell wall integrity response. Five Cup9-activated surface-protein genes have roles in cell wall integrity, based on mutant analysis published previously (*PGA31* and *IFF11*) or shown here (*ORF19.3499*, *ORF19.851*, or *PGA28*), and therefore may explain the hypersensitivity of the *cup9*Δ/Δmutant to caspofungin. Our findings define Cup9 as a new determinant of caspofungin susceptibility.

Keywords: *Candida*; cell wall integrity; regulation

Introduction

The fungus *Candida albicans* causes diverse infections with considerable morbidity and mortality. Invasive infections cause 10,000 deaths per year in the United States, and an estimated 400,000 deaths per year worldwide (Brown *et al.* 2012). The organism remains a threat for many reasons, including the limited selection and scope of antifungal drugs, the occurrence of drug resistance, the ability of the organism to grow as biofilm, and the diverse virulence determinants that enable it to infect almost any tissue (Mayer *et al.* 2013; Robbins *et al.* 2016).

This organism can grow in several morphological forms, notably yeast and hyphae (Sudbery 2011). Yeast-form cells, also called blastospores, are ovoid cells that grow by budding. Hyphae are filaments that have contiguous cell compartments separated by septa and grow by tip extension. Hyphae are closely tied to pathogenesis (Mayer *et al.* 2013) because (i) hyphae are prominent in infected tissue; (ii) hyphal growth is induced *in vitro* by infection-relevant conditions, such as 37°C temperature, neutral pH, and presence of serum; (iii) many genes that are highly induced in hyphae are required for pathogenicity; and (iv) all *C. albicans* mutant strains that are defective in hyphal formation in vivo are also defective in pathogenesis. Although there are other types of pathogenesis-defective mutants (Mayer *et al.* 2013), the program of hyphal formation is widely considered a major determinant of *C. albicans* pathogenicity during invasive infection.

C. albicans is surrounded by a cell wall that is the target of echinocandin antifungals, such as caspofungin, as well as several other therapeutic strategies under development (Robbins *et al.* 2016). The organism's response to stress caused by cell wall inhibitors enables it to survive echinocandin treatment (Ernst and Pla 2011). Defects in known cell wall stress response regulators, such as conserved Cell Wall Integrity MAP Kinase pathway components (Dichtl *et al.* 2016), cause *C. albicans* to become hypersensitive to echinocandins.

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The transcriptional circuitry that governs the cell wall stress response has been characterized in considerable detail (Heredia *et al.* 2020a). Three key transcription factors—Cas5, Rlm1, and Sko1—were identified as insertion mutations that caused hypersensitivity to caspofungin. Cas5 has the broadest role among them in that it controls the largest number of genes, including over half of caspofungin-responsive genes (Bruno *et al.* 2006; Xie *et al.* 2017; Heredia *et al.* 2020a). Cas5 is also required for normal cell division and numerous stress responses (Xie *et al.* 2017; Heredia *et al.* 2020a). Sko1 and Rlm1 control fewer caspofungin-responsive genes, and are interconnected in that Rlm1 is required for induction of Sko1 RNA by caspofungin (Bruno *et al.* 2006; Rauceo *et al.* 2008; Heredia *et al.* 2020a, 2020b).

The yeast and hyphal growth forms differ considerably in cell wall constituents (Heilmann *et al.* 2011; Sudbery 2011; Hall 2015). For example, the highly expressed yeast-specific gene *YWP1* encodes a cell wall protein; the highly expressed hyphal-specific genes *ALS3*, *HWP1*, and *HYR1* encode cell wall proteins. On this basis, we suspected that the cell wall stress response may differ between yeast and hyphae. To date, the response to cell wall stress has been characterized primarily in yeast-form *C. albicans* cells (see, for example, Xie *et al.* 2017; Heredia *et al.* 2020a, 2020b). In this study, we characterize the cell wall stress response under hyphal growth conditions. Our findings provide evidence for an environmentally contingent response, though not a hyphal-specific response, that is mediated in part by the transcription factor Cup9.

Materials and methods

Media

Strains were grown in YPD (2% Bacto peptone, 2% dextrose, and 1% yeast extract), YPD with 25 mM HEPES or in RPMI-1640 with L-glutamine and 25 mM HEPES, without sodium bicarbonate (R4130 SIGMA). YPD with 25 mM HEPES and RPMI-1640 medium was adjusted to pH7.4 using NaOH. Transformants were selected on synthetic medium (2% dextrose, 1.7% Difco yeast nitrogen base with ammonium sulfate and auxotrophic supplements) or on YPD + 400 μg/ml nourseothricin [clonNAT, WERNER BioAgents] for nourseothricin-resistant isolates.

The medium for the mutant screen was RPMI+serum+caspofungin, buffered with $NaHCO_3$ to prevent pH changes from a 5% CO_2 atmosphere. One bottle of RPMI powder (R4130-10X*1L WITH BUFFER; Sigma) was mixed well with 20 g of Bacto-Agar and 876 ml of deionized water, then autoclaved with a stir bar in place. These components were added after autoclaving with mixing: 100 ml of Fetal Bovine Serum (S11150H; Atlanta Biologicals), 24 ml of filter sterilized 1 M Sodium Bicarbonate solution, and 0.120 ml of 250 μg/ml caspofungin stock solution. Plates were poured and allowed to solidify and dry before use.

Strains

All *C. albicans* strains used in this study are listed in Supplementary Table S1, and primer sequences are listed in Supplementary Table S2.

The *C. albicans cup9* deletion mutant (mutant no. 991; *orf19.6514*) was obtained from the Noble homozygous deletion library (Homann *et al.* 2009) at the Fungal Genetics Stock Center. For complementation of the *cup9*Δ/Δ mutant, PCR primers were designed to amplify genomic DNA of strain SC5314 from 3 kb upstream to 0.5 kb downstream of the open reading frame (ORF). These primers (called CUP9_for and CUP9_rev) have 40 b flanking sequences with homology to pSN105+NruI. pSN105+NruI is a derivative of pSN105 (Noble *et al.* 2010) with a NruI site inserted right next to the *LEU2* cassette. To construct a complementing plasmid containing the *CUP9* gene, the resulting PCR product was co-transformed into *S. cerevisiae* with Nru1-digested pSN105+NruI. The resulting plasmid DNA was digested with HpaI and integrated at the native *CUP9* locus (2.4 kb upstream of the ORF). pSN105 and pSN105+NruI digested with PmeI were transformed into WT (SN250) and the mutant strain respectively to generate prototrophic, marker-matched strains.

The *C. albicans orf19.3499*, *orf19.851*, and *pga28* deletion mutants were constructed in the SN250 strain background through the transient CRISPR-Cas9 system (Min *et al.* 2016). The gene deletion construct was synthesized using the *C.d. ARG4* plasmid pSN69 (Noble and Johnson 2005). The sgRNA cassette, the *CaCAS9* expression cassette and the deletion construct were co-transformed into strain SN250 (Noble and Johnson 2005). PCR assays for each wild-type allele were accomplished with the "Gene Name for" and "Gene Name rev" primers indicated in Supplementary Table S2.

The *orf19.851*Δ/Δ *pga28*Δ/Δ *orf19.3499*Δ/Δ triple mutant was constructed using the transient CRISPR-Cas9 system (Min *et al.* 2016) in strain SN152. The deletion markers were *C.d. ARG4* from pSN69 for *ORF19.851*, *C.d. HIS1* from pSN52 for *PGA28*, and *C.m. LEU2* from pSN40 for *ORF19.3499* (Noble and Johnson 2005). The triple mutant was constructed sequentially by co-transforming a deletion marker construct, the *CaCAS9* expression cassette and the appropriate sgRNA for the gene being targeted. Transformants were selected on drop out medium and screened by colony PCR for homozygous deletions of the targeted gene. The order of deletion was first *orf19.851::ARG4*, then *pga28::HIS1*, and finally *orf19.3499::LEU2*.

The *efg1*Δ/Δ and *cup9*Δ/Δ *efg1*Δ/Δ deletion mutants were constructed using the transient CRISPR-Cas9 system (Min *et al.* 2016). The *NAT* marker from pNAT (Min *et al.* 2016) was used as the deletion marker for *EFG1*. The sgRNA cassette (targeting *EFG1*), the *CaCAS9* expression cassette, and the *NAT* deletion construct were co-transformed into strain SN250 (*CUP9*) and YI192 (*cup9*Δ/Δ) and transformants were selected on YPD + nourseothricin. NAT resistant transformants were checked by colony PCR for homozygous deletion strains.

The *efg1*Δ/Δ and *cup9*Δ/Δ *efg1*Δ/Δ strains that had *EFG1* reconstituted were constructed using the transient CRISPR-Cas9 system (Min *et al.* 2016). An *EFG1* cassette was amplified from wild type (SC5314) genomic DNA and co-transformed with an *ARG4* cassette (also amplified from wild type genomic DNA), the *CaCAS9* expression cassette and 2 sgRNA cassettes: one for targeting *efg1*Δ*::NAT* and one for targeting the *arg4::dpl200* junction found in the SN250 strain background. Transformants were selected for growth on CSM-arg medium and then screened for nourseothricin sensitivity. Arg^+ NAT^S strains were checked by colony PCR for the absence of *arg4::dpl200* sequences and absence of *NAT* sequences to identify homozygous *ARG/ARG EFG1/EFG1* strains. To distinguish *EFG1/EFG1* reconstituted strains from *EFG1/EFG1* wild-type strains we designate the reconstituted genotype "*efg1*(Δ/Δ)+/+."

Spot dilution assay

Strains were grown overnight in 5 ml YPD medium at 30°C. Cell density was measured at OD_{600} for each strain, which were then diluted to an OD_{600} of 3.0 in H_2O. Fivefold dilutions were made of the OD_{600} 3.0 stock and these were plated on the indicated media. Plates were incubated at 30°C or 37°C (as indicated in figure legends) for 2–4 days.

Microscopy

Cells from an overnight 5 ml YPD 30°C culture were washed with 5 ml H_2O and re-suspended again in 5 ml H_2O. The cell suspension was diluted to an $OD_{600} = 20$ in H_2O and finally diluted 1:100 into 5 ml RPMI medium. Cells were grown at 30°C with rotating for 6 hours and visualized with a Zeiss Axio Observer Z.1 fluorescence microscope and a 20x DIC objective.

RNA collection for RNA sequencing

For RNA sequencing, wild type (SC5314) and *cup9*Δ/Δ (YI192) cells from an overnight 5 ml YPD 30°C culture were washed with 5 ml H_2O and re-suspended again in 5 ml H_2O. The cell suspension was diluted to an $OD_{600} = 20$ in H_2O and finally diluted 1:100 into 25 ml RPMI medium. Cells were grown at 37°C with 200 rpm shaking for 2 hours. Caspofungin was then added to a final concentration of 27.5 ng/ml, and the control cultures received an equal volume of water. Cells were collected by filtration 30 minutes after drug administration. RNA extractions were performed using Qiagen RNeasy mini kit (Cat#74104) with modifications as described previously (Xu *et al.* 2015).

RNA sequencing

RNA-seq libraries (nonstrand-specific and paired end) were prepared with the TruSeq RNA Sample Prep kit (Illumina). The total RNA samples were subject to poly(A) enrichment as part of the TruSeq protocol. In total, 150 nt of sequence was determined from both ends of each cDNA fragment using the HiSeq platform (Illumina). Sequencing reads were aligned to the *C. albicans* reference (Assembly A21) using TopHat2 (Kim *et al.* 2013). The alignment files from TopHat2 were used to generate read counts for each gene and a statistical analysis of differential gene expression was performed using the DEseq package from Bioconductor (Anders and Huber 2010). Each experimental group consisted of two biological replicates.

Data availability

Raw sequencing reads (RNA-seq) from this study have been submitted to the NCBI sequence read archive (SRA) under BioProject accession number PRJNA693694. The data are also available upon request. All other data are within the manuscript and its Supplementary materials files. Supplementary material is available at figshare: https://doi.org/10.25386/genetics.14568864.

Results

Identification of mutants hypersensitive to caspofungin under hyphal growth conditions

We hypothesized that some cell wall integrity regulators may affect caspofungin susceptibility in only yeast or hyphae. To explore this idea we sought to assay caspofungin susceptibility under hyphal inducing conditions. In pilot studies, we found that caspofungin susceptibility was difficult to assay in RPMI medium at 37°C, a typical hyphal growth condition. Therefore, we conducted assays in RPMI medium at 30°C, a condition in which hyphal growth of wild-type strain CW542 occurs (Supplementary Figure S1).

As an initial test of this hypothesis, we assayed caspofungin susceptibility of 27 previously identified cell wall integrity regulatory mutants in RPMI medium at 30°C. Susceptibility to cell wall inhibitors had been tested previously for these strains mainly under yeast growth conditions (Bruno *et al.* 2006; Rauceo *et al.* 2008; Blankenship *et al.* 2010; Roman *et al.* 2015; Heredia *et al.* 2020b). All 27 mutants were hypersensitive to caspofungin in RPMI medium at 30°C as well (Supplementary Figure S2). These results indicate that all of these regulators govern cell wall integrity under these growth conditions.

As a second test of this hypothesis, we screened 165 transcription factor deletion mutants (Homann *et al.* 2009) for sensitivity to caspofungin under hyphal growth conditions. Each strain was tested by a spot-dilution assay on RPMI + 10% serum + 30 ng/ml caspofungin in a 5% CO_2 atmosphere at 37°C. Mutants that presented a caspofungin-hypersensitive phenotype included *cas5*Δ/Δ, *cup9*Δ/Δ, *swi4*Δ/Δ, *mig1*Δ/Δ, *zcf14*Δ/Δ, *bas1*Δ/Δ, *sef1*Δ/Δ, *fgr15*Δ/Δ, *dal81*Δ/Δ, *orf19.2961*Δ/Δ, and *czf1*Δ/Δ. Cell wall integrity defects have been reported previously for the mutants cas5Δ/Δ (Bruno *et al.* 2006; Xie *et al.* 2017), *mig1*Δ/Δ (Lagree *et al.* 2020), *czf1*Δ/Δ (Mottola *et al.* 2021), and *swi4*Δ/Δ (Xie *et al.* 2017). Therefore, this screen identified several new mutants that are hypersensitive to caspofungin.

We focused on Cup9 in the present study because of the strength of the mutant phenotype (Figure 1A) and early indications that the mutant might cause a cell wall integrity defect only in hyphae (presented below). Cup9 has been characterized previously as a negative regulator of hyphal formation (Homann *et al.* 2009; Guan *et al.* 2013; Lu *et al.* 2014; Meir *et al.* 2018), but to our knowledge has not been tied previously to cell wall integrity. A complemented strain, made by introducing a wild-type copy of *CUP9* into the mutant, showed greatly improved growth in the presence of caspofungin (Figure 1A, *cup9*Δ/Δ/+). Complementation was evident

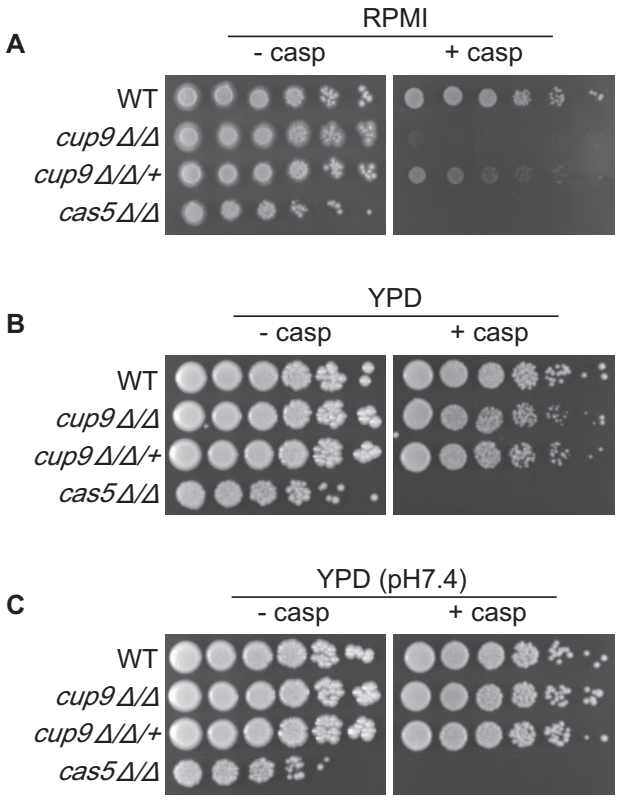

Figure 1 Effect of growth conditions on *cup9*Δ/Δ mutant caspofungin hypersensitivity. The wild-type strain (CW542), *cas5*Δ/Δ caspofungin hypersensitive control (VIC1147), *cup9*Δ/Δ mutant (YI192), and *cup9*Δ/Δ/+ complemented strain (YI243) were serially diluted onto RPMI (A), YPD (B), or YPD buffered at pH7.4 (C) with or without 125 ng/ml caspofungin. Cells were incubated for 3 days at 30°C.

in RPMI medium at 30°C (Figure 1A) or 37°C (Supplementary Figure S3). We confirmed that the *cup9*Δ/Δ mutant grows as hyphae in RPMI (Supplementary Figure S1). The complementation test verified that the *CUP9* deletion causes caspofungin hypersensitivity under hyphal growth conditions.

The *cup9*Δ/Δ mutant showed a comparably mild caspofungin-hypersensitive phenotype in YPD medium at 30°C, a yeast growth condition (Figure 1B). RPMI and YPD differ in pH, a possible cause of differential drug sensitivity. However, in YPD buffered at pH 7.4 (the pH of RPMI), the *cup9*Δ/Δ mutant remained only mildly caspofungin-hypersensitive (Figure 1C). These results show that the role of Cup9 in cell wall integrity is contingent upon growth conditions.

Caspofungin hypersensitivity of a yeast-form *cup9*Δ/Δ mutant

If Cup9 governs caspofungin sensitivity most strongly in the hyphal growth form, then a mutation that blocks hyphal growth may suppress the *cup9*Δ/Δ phenotype. We tested this prediction with a mutation in *EFG1*, which specifies a transcriptional regulator of hyphal development. An *efg1*Δ/Δ mutant is defective in hyphal-specific gene expression and hyphal morphogenesis (Lo *et al.* 1997; Stoldt *et al.* 1997; Braun and Johnson 2000; Sudbery 2011). We created a *cup9*Δ/Δ *efg1*Δ/Δ double deletion mutant and its *EFG1/EFG1* reconstituted strain. Loss of *EFG1* suppressed caspofungin hypersensitivity of the *cup9*Δ/Δ mutant (Figure 2, *cup9*Δ/Δ *efg1*Δ/Δ). Reconstitution of *EFG1* restored caspofungin hypersensitivity [Figure 2, *cup9*Δ/Δ *efg1*(Δ/Δ)+/+]. This result is consistent with the model that Cup9 is required for cell wall integrity in hyphal cells but not in yeast-form cells. However, we also tested caspofungin sensitivity of an *efg1*Δ/Δ mutant made in a *CUP9*+/+ background on RPMI medium (Figure 2). The *efg1*Δ/Δ mutant (Figure 2, *efg1*Δ/Δ) showed less sensitivity than the *cup9*Δ/Δ *efg1*Δ/Δ mutant, and introduction of wild-type *EFG1* alleles into the mutant increased caspofungin sensitivity [Figure 2, *efg1*(Δ/Δ)+/+]. Because the loss of *CUP9* increases caspofungin sensitivity in an *efg1*Δ/Δ background, the role of Cup9 in cell wall integrity is not hyphal-specific; rather it is environmentally contingent.

Transcriptional response to caspofungin during hyphal growth

To determine the gene expression response to caspofungin in hyphal growth conditions, we conducted an RNA-seq comparison of the wild-type strain SC5314 with or without caspofungin treatment in RPMI at 37°C. We chose this temperature rather than 30°C because 37°C is very widely used to promote hyphal growth. In the wild type, 361 genes were up-regulated at least twofold and 145 genes were down regulated at least twofold after caspofungin treatment (Supplementary Table S3). Both up- and down-regulated genes were enriched significantly for GO descriptors related to the cell surface, as expected if the response alters the cell wall.

Genes regulated by Cup9 were identified through comparison of RNA-seq data of the *cup9*Δ/Δ mutant and the wild type, both treated with caspofungin. Among 361 genes that were up-regulated in the wild type, 40 genes were expressed at lower levels in the *cup9*Δ/Δ mutant (Table 1). This gene set showed some enrichment for cell surface-related functions. Among 145 genes that were down-regulated in the wild type, 8 genes were expressed at higher levels in the *cup9*Δ/Δ mutant (Table 1). These genes were enriched for zinc homeostasis functions. An additional 65 genes showed altered expression in the *cup9*Δ/Δ mutant compared to wild type, both treated with caspofungin (Supplementary Table S3). This gene set was not enriched significantly for any Gene Ontology terms. Overall, the narrow gene expression impact of a *CUP9* defect suggests that Cup9 is not a global regulator of cell wall integrity.

Cup9-regulated genes related to cell wall integrity

Among caspofungin-induced Cup9-regulated genes, 26 encode possible secreted proteins or cell wall proteins based on presence of a signal peptide sequence (Table 1). Two genes in this group, *PGA31* and *IFF11*, have been shown to be required for cell wall integrity (Bates *et al.* 2007; Plaine *et al.* 2008). We hypothesized that reduced expression of other potential secreted/cell wall protein genes may also contribute to the sensitivity of the *cup9*Δ/Δ mutant to caspofungin.

To see if Cup9-regulated secreted/cell wall protein genes may contribute to cell wall integrity, we tested three less characterized cell surface protein genes *ORF19.3499*, *ORF19.851*, and *PGA28*. Deletion mutants *orf19.3499*Δ/Δ, *orf19.851*Δ/Δ, and *pga28*Δ/Δ were slightly sensitive to caspofungin (Figure 3). No synergy was evident in a triple deletion mutant (Figure 3). These results suggest that *ORF19.3499*, *ORF19.851*, and *PGA28* have a role in cell wall integrity. The relatively mild mutant phenotypes may indicate that the gene products have a minor role, or that their functions overlap with those of other Cup9-regulated genes.

Discussion

We have characterized a new regulator of the *C. albicans* cell wall stress response, Cup9. The rationale that led us to Cup9 was that

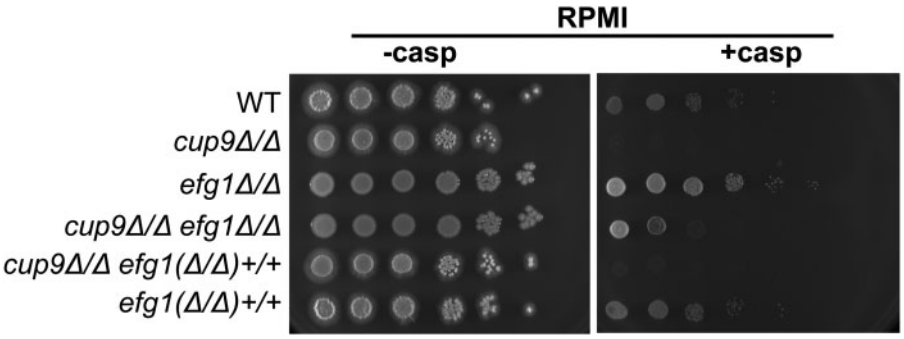

Figure 2 Effect of an *efg1*Δ/Δ mutation on *cup9*Δ/Δ mutant caspofungin hypersensitivity. The wild-type strain (CW1757), *cup9*Δ/Δ mutant (YI192), *efg1*Δ/Δ mutant (CW1792), *cup9*Δ/Δ *efg1*Δ/Δ mutant (CW1796), *cup9*Δ/Δ *efg1*(Δ/Δ)+/+ reconstituted strain (CW1785), and *efg1*(Δ/Δ)+/+ reconstituted strain (CW1779) were serially diluted onto RPMI with or without 125 ng/ml caspofungin. Cells were incubated for 4 days at 30°C.

Table 1 Genes that respond in parallel to caspofungin and Cup9

ORF ID	Gene symbol	Fold change Wt +caspo/ Wt—caspo	Fold change cup9 +caspo/Wt +caspo	Signal peptide
orf19.2034	—	>60	0.06	—
orf19.3499	—	54.85	0.17	x
orf19.6486	*LDG3*	48.32	0.15	x
orf19.937	—	24.55	0.28	x
orf19.5302	*PGA31*	23.66	0.41	x
orf19.5303	*PGA30*	18.41	0.33	x
orf19.1539	—	17.30	6.26	—
orf19.36.1	—	17.16	0.25	—
orf19.1171	—	14.92	0.43	x
orf19.5399	*IFF11*	13.92	0.37	x
orf19.5874	—	13.16	0.11	x
orf19.4082	*DDR48*	12.36	0.48	—
orf19.1524	*SPR3*	9.82	0.31	—
orf19.2160	*NAG4*	8.74	0.40	—
orf19.6475	—	7.45	0.31	x
orf19.4688	*DAG7*	7.42	0.32	x
orf19.7170	—	7.28	0.35	x
orf19.2252	—	6.27	0.12	—
orf19.2809	*CTN3*	5.48	0.50	—
orf19.1048	*IFD6*	5.14	0.37	—
orf19.2593	*BIO2*	4.92	0.38	—
orf19.3643	—	4.50	0.33	—
orf19.1440.1	—	4.26	0.41	—
orf19.5975	—	3.71	0.36	—
orf19.4255	*ECM331*	2.94	0.42	x
orf19.938	—	2.89	0.43	—
orf19.4934	*OP4*	2.76	0.44	x
orf19.1440.2	—	2.60	0.43	—
orf19.24	*RTA2*	2.57	0.34	—
orf19.3710	*YHB5*	2.48	0.46	—
orf19.3375	—	2.43	0.38	x
orf19.555	—	2.41	0.16	x
orf19.1868	*RNR22*	2.39	0.49	—
orf19.6837	*FMA1*	2.35	0.44	—
orf19.4936.1	—	2.28	0.47	—
orf19.851	—	2.17	0.24	x
orf19.5542	*SAP6*	2.09	0.22	x
orf19.17	*SCP1*	2.08	0.43	—
orf19.5144	*PGA28*	2.03	0.22	x
orf19.7557	*FGR46*	2.02	0.44	x
orf19.3794	*ZAP1/CSR1*	0.48	2.85	—
orf19.1930	*CFL5*	0.45	3.20	x
orf19.5141	—	0.35	4.27	x
orf19.3895	*CHT2*	0.24	8.74	x
orf19.6142	—	0.21	7.08	—
orf19.3112	*ZRT1*	0.17	2.44	x
orf19.3111	*PRA1*	0.16	2.83	x
orf19.2038	—	0.15	3.61	—

Genes that are caspofungin-induced and Cup9-activated or caspofungin-repressed and Cup9-repressed are listed. Complete differentially expressed gene analysis is in Supplementary Table S3. Genes whose products have a predicted signal peptide are indicated.

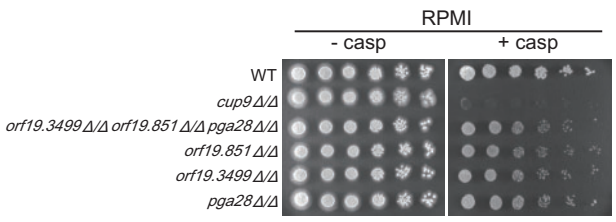

Figure 3 Roles of Cup9-regulated genes in cell wall integrity. Comparisons of deletion mutants were conducted. The wild-type strain (CW542), *orf19.3499*Δ/Δ *orf19.851*Δ/Δ *pga28*Δ/Δ triple mutant (CW1697), *orf19.851*Δ/Δ mutant (YI459), *orf19.3499*Δ/Δ mutant (YI463), *pga28*Δ/Δ mutant (YI464), and *cup9*Δ/Δ mutant (YI192), were serially diluted onto RPMI solid medium with or without 125 ng/ml caspofungin. Cells were incubated for 3 days at 30°C.

distinct regulatory functions may be required in hyphal cells as compared to yeast cells, given that their cell wall protein constituents are different. However, our results instead argue that Cup9 is required for cell wall integrity under hyphal growth conditions, but that Cup9 has a functional role in cell wall integrity even in yeast form cells. Therefore, it is environmental conditions rather than cell type that determines the need for Cup9 in cell wall integrity.

Cup9 has been characterized previously as a negative regulator of hyphal formation. For example, a *cup9*Δ/Δ mutant displays increased filamentation in various *in vitro* growth assays (Homann *et al.* 2009; Guan *et al.* 2013). Mechanistic insight into Cup9 function has come from analysis of its roles in farnesol inhibition of hyphal formation (Lu *et al.* 2014) and oral mucosal

colonization (Meir *et al.* 2018). As a mediator of hyphal inhibition by farnesol, a quorum-sensing molecule, Cup9 functions as a repressor of *SOK1* RNA accumulation; Sok1 in turn promotes degradation of the hyphal repressor Nrg1. Farnesol prevents Cup9 protein degradation (Lu *et al.* 2014). Therefore, farnesol promotes *SOK1* repression, resulting in increased Nrg1 accumulation. The role of Cup9 in oral colonization is at least in part tied to its impact on hyphal formation. The *cup9*Δ/Δ mutant displays much more prominent hyphae in infected tongue samples than the wild-type strain (Meir *et al.* 2018). RNA-seq comparison of *cup9*Δ/Δ and wild-type cells grown on a semisolid surface at 37°C shows that Cup9 represses expression of some key hyphal-associated genes, including *HWP1* and *ECE1* (Meir *et al.* 2018). Thus, the previously known biological roles of Cup9 are tied to its negative regulation of hyphal formation.

Three observations argue that the impact of Cup9 on the cell wall stress response is independent of its regulation of hyphal formation. First, caspofungin hypersensitivity of the *cup9*Δ/Δ mutant is evident under hyphal-inducing conditions, when wild-type, mutant, and complemented strains all grow as hyphae. Second, in a hyphal-defective *efg1*Δ/Δ mutant background, a *cup9*Δ/Δ mutation causes increased caspofungin sensitivity. Third, little increased expression of hyphal-associated genes, except for a twofold increase in *SOD5* RNA levels, is evident in the *cup9*Δ/Δ mutant under the hyphal-inducing conditions we used for RNA-seq analysis. These observations, together with previous work, suggest that Cup9 has two functions: it represses hyphal formation, and it promotes a response to cell wall damage.

Environmental conditions govern the functional activity of Cup9. This idea is supported by our observation that the *cup9*Δ/Δ mutant's caspofungin hypersensitivity depends upon the growth medium. The idea is further supported by comparison of gene expression data for *cup9*Δ/Δ mutants. In the farnesol study, Lu *et al.* (2014) showed that a *cup9*Δ/Δ mutant has elevated *SOK1* expression when grown in YPD+farnesol liquid medium at 37°C. In the oral colonization study, Meir *et al.* (2018) showed that a *cup9*Δ/Δ mutant has elevated *HWP1* and *ECE1* expression when grown on Todd–Hewitt solid medium at 37°C, but did not detect significantly elevated *SOK1* expression. In our present study, the *cup9*Δ/Δ mutant did not cause increased *SOK1*, *HWP1*, or *ECE1* expression in RPMI+caspofungin liquid medium at 37°C. In fact, only 10 genes display parallel regulation by Cup9 in our study and the analysis of Meier et al. (Meir *et al.* 2018): *WOR1* and *DAG7* are down-regulated in the mutant; *SOD5, PGA26, PRA1, GIT1, orf19.4450.1, orf19.6586, MNN1*, and *CHT2* are up-regulated in the mutant. The observations above suggest that environmental conditions govern the gene set that is under Cup9 control. This relationship between environmental conditions and Cup9-responsive genes may explain why the *cup9*Δ/Δ mutant has environmentally contingent phenotypes.

How may the environment influence Cup9 activity? We note that Cup9 has a helix-turn-helix or homeodomain DNA binding domain, and such proteins often have binding partners that modify their binding specificity (Burglin and Affolter 2016). A simple model is that Cup9 has a few different environmentally responsive binding partners, and each directs it to act on a different gene set.

How does Cup9 promote cell wall integrity? We propose that several Cup9-responsive genes contribute to this process. Prior studies have shown that mutation of *PGA31* or *IFF11* confer hypersensitivity to cell wall inhibitors (Bates *et al.* 2007; Plaine *et al.* 2008). Both of these genes are induced by caspofungin and are expressed at reduced levels in a *cup9*Δ/Δ mutant. Three additional genes studied here—*ORF19.3499*, *ORF19.851*, and *PGA28*—share these regulatory features and mutant phenotypes, though mutation of any one or all three of those genes causes fairly mild caspofungin hypersensitivity. These observations argue that Cup9 promotes cell wall integrity by activating expression of several genes that encode cell wall- or surface-localized proteins, each of which contributes to overall cell wall integrity.

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Conflicts of interest

None declared.

Literature cited

Anders S, Huber W. 2010. Differential expression analysis for sequence count data. Genome Biol. 11:R106.

Bates S, de la Rosa JM, MacCallum DM, Brown AJ, Gow NA, *et al.* 2007. *Candida albicans* Iff11, a secreted protein required for cell wall structure and virulence. Infect Immun. 75:2922–2928.

Blankenship JR, Fanning S, Hamaker JJ, Mitchell AP. 2010. An extensive circuitry for cell wall regulation in *Candida albicans*. PLoS Pathog. 6:e1000752.

Braun BR, Johnson AD. 2000. *TUP1, CPH1* and *EFG1* make independent contributions to filamentation in *Candida albicans*. Genetics. 155:57–67.

Brown GD, Denning DW, Gow NA, Levitz SM, Netea MG, *et al.* 2012. Hidden killers: human fungal infections. Sci Transl Med. 4:165rv113.

Bruno VM, Kalachikov S, Subaran R, Nobile CJ, Kyratsous C, *et al.* 2006. Control of the *C. albicans* cell wall damage response by transcriptional regulator Cas5. PLoS Pathog. 2:e21.

Burglin TR, Affolter M. 2016. Homeodomain proteins: an update. Chromosoma. 125:497–521.

Dichtl K, Samantaray S, Wagener J. 2016. Cell wall integrity signalling in human pathogenic fungi. Cell Microbiol. 18:1228–1238.

Ernst JF, Pla J. 2011. Signaling the glycoshield: maintenance of the *Candida albicans* cell wall. Int J Med Microbiol. 301:378–383.

Guan G, Xie J, Tao L, Nobile CJ, Sun Y, *et al.* 2013. Bcr1 plays a central role in the regulation of opaque cell filamentation in *Candida albicans*. Mol Microbiol. 89:732–750.

Hall RA. 2015. Dressed to impress: impact of environmental adaptation on the *Candida albicans* cell wall. Mol Microbiol. 97:7–17.

Heilmann CJ, Sorgo AG, Siliakus AR, Dekker HL, Brul S, *et al.* 2011. Hyphal induction in the human fungal pathogen *Candida albicans*

reveals a characteristic wall protein profile. Microbiology (Reading). 157:2297–2307.

Heredia MY, Gunasekaran D, Ikeh MAC, Nobile CJ, Rauceo JM. 2020a. Transcriptional regulation of the caspofungin-induced cell wall damage response in *Candida albicans*. Curr Genet. 66:1059–1068.

Heredia MY, Ikeh MAC, Gunasekaran D, Conrad KA, Filimonava S, *et al.* 2020b. An expanded cell wall damage signaling network is comprised of the transcription factors Rlm1 and Sko1 in *Candida albicans*. PLoS Genet. 16:e1008908.

Homann OR, Dea J, Noble SM, Johnson AD. 2009. A phenotypic profile of the *Candida albicans* regulatory network. PLoS Genet. 5:e1000783.

Kim D, Pertea G, Trapnell C, Pimentel H, Kelley R, *et al.* 2013. TopHat2: accurate alignment of transcriptomes in the presence of insertions, deletions and gene fusions. Genome Biol. 14:R36.

Lagree K, Woolford CA, Huang MY, May G, McManus CJ, *et al.* 2020. Roles of *Candida albicans* Mig1 and Mig2 in glucose repression, pathogenicity traits, and *SNF1* essentiality. PLoS Genet. 16: e1008582.

Lo HJ, Kohler JR, DiDomenico B, Loebenberg D, Cacciapuoti A, *et al.* 1997. Nonfilamentous *C. albicans* mutants are avirulent. Cell. 90: 939–949.

Lu Y, Su C, Unoje O, Liu H. 2014. Quorum sensing controls hyphal initiation in *Candida albicans* through Ubr1-mediated protein degradation. Proc Natl Acad Sci USA. 111:1975–1980.

Mayer FL, Wilson D, Hube B. 2013. *Candida albicans* pathogenicity mechanisms. Virulence. 4:119–128.

Meir J, Hartmann E, Eckstein MT, Guiducci E, Kirchner F, *et al.* 2018. Identification of *Candida albicans* regulatory genes governing mucosal infection. Cell Microbiol. 20:e12841.

Min K, Ichikawa Y, Woolford CA, Mitchell AP. 2016. *Candida albicans* gene deletion with a transient CRISPR-Cas9 system. mSphere. 1: e00130-16.

Mottola A, Ramírez-Zavala B, Hünniger K, Kurzai O, Morschhäuser J. 2021. The zinc cluster transcription factor Czf1 regulates cell wall architecture and integrity in *Candida albicans*. Mol Microbiol. doi: 10.1111/mmi.14727

Noble SM, French S, Kohn LA, Chen V, Johnson AD. 2010. Systematic screens of a *Candida albicans* homozygous deletion library decouple morphogenetic switching and pathogenicity. Nat Genet. 42: 590–598.

Noble SM, Johnson AD. 2005. Strains and strategies for large-scale gene deletion studies of the diploid human fungal pathogen *Candida albicans*. Eukaryot Cell. 4:298–309.

Plaine A, Walker L, Da Costa G, Mora-Montes HM, McKinnon A, *et al.* 2008. Functional analysis of *Candida albicans* GPI-anchored proteins: roles in cell wall integrity and caspofungin sensitivity. Fungal Genet Biol. 45:1404–1414.

Rauceo JM, Blankenship JR, Fanning S, Hamaker JJ, Deneault JS, *et al.* 2008. Regulation of the *Candida albicans* cell wall damage response by transcription factor Sko1 and PAS kinase Psk1. Mol Biol Cell. 19:2741–2751.

Robbins N, Wright GD, Cowen LE. 2016. Antifungal drugs: the current armamentarium and development of new agents. Microbiol Spectr. 4.doi:10.1128/microbiolspec.FUNK-0002-2016.

Roman E, Alonso-Monge R, Miranda A, Pla J. 2015. The Mkk2 MAPKK regulates cell wall biogenesis in cooperation with the Cek1-pathway in *Candida albicans*. PLoS One. 10:e0133476.

Stoldt VR, Sonneborn A, Leuker CE, Ernst JF. 1997. Efg1p, an essential regulator of morphogenesis of the human pathogen *Candida albicans*, is a member of a conserved class of bHLH proteins regulating morphogenetic processes in fungi. EMBO J. 16: 1982–1991.

Sudbery PE. 2011. Growth of *Candida albicans* hyphae. Nat Rev Microbiol. 9:737–748.

Xie JL, Qin L, Miao Z, Grys BT, Diaz JC, *et al.* 2017. The *Candida albicans* transcription factor Cas5 couples stress responses, drug resistance and cell cycle regulation. Nat Commun. 8:499.

Xu W, Solis NV, Ehrlich RL, Woolford CA, Filler SG, *et al.* 2015. Activation and alliance of regulatory pathways in *C. albicans* during mammalian infection. PLoS Biol. 13:e1002076.

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