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# Disruption of the Transcriptional Regulator Cas5 Results in Enhanced Killing of *Candida albicans* by Fluconazole

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**Azole antifungal agents such as fluconazole exhibit fungistatic activity against *Candida albicans*. Strategies to enhance azole antifungal activity would be therapeutically appealing. In an effort to identify transcriptional pathways that influence the killing activity of fluconazole, we sought to identify transcription factors (TFs) involved in this process. From a collection of *C. albicans* strains disrupted for genes encoding TFs (O. R. Homann, J. Dea, S. M. Noble, and A. D. Johnson, PLoS Genet. 5:e1000783, 2009, http://dx.doi.org/10.1371/journal.pgen.1000783), four strains exhibited marked reductions in minimum fungicidal concentration (MFCs) in both RPMI and yeast extract-peptone-dextrose (YPD) media. One of these genes, *UPC2*, was previously characterized with regard to its role in azole susceptibility. Of mutants representing the three remaining TF genes of interest, one (*CAS5*) was unable to recover from fluconazole exposure at concentrations as low as 2 μg/ml after 72 h in YPD medium. This mutant also showed reduced susceptibility and a clear zone of inhibition by Etest, was unable to grow on solid medium containing 10 μg/ml fluconazole, and exhibited increased susceptibility by time-kill analysis. *CAS5* disruption in highly azole-resistant clinical isolates exhibiting multiple resistance mechanisms did not alter susceptibility. However, *CAS5* disruption in strains with specific resistance mutations resulted in moderate reductions in MICs and MFCs. Genome-wide transcriptional analysis was performed in the presence of fluconazole and was consistent with the suggested role of *CAS5* in cell wall organization while also suggesting a role in iron transport and homeostasis. These findings suggest that Cas5 regulates a transcriptional network that influences the response of *C. albicans* to fluconazole. Further delineation of this transcriptional network may identify targets for potential cotherapeutic strategies to enhance the activity of the azole class of antifungals.**

*Candida albicans* is the most prevalent opportunistic human fungal pathogen, causing mucosal, cutaneous, and systemic infections, including oropharyngeal candidiasis (OPC), which is the most common opportunistic infection among AIDS patients (1, 2). *Candida* species collectively are also the fourth leading cause of nosocomial infections and are associated with high mortality rates (3, 4). The azole antifungals, particularly fluconazole (FLC), are the most widely used antifungals for treatment of *Candida* infections (1). However, *C. albicans* exhibits inhibited growth in the presence of azole antifungals; thus, these agents are fungistatic against this organism (5). Identifying strategies to impart enhanced killing activity to the azoles could improve their efficacy against *C. albicans* and may also diminish the development of resistance.

In *C. albicans*, inhibition of the ergosterol biosynthesis pathway is not lethal (5). It is possible that specific stress responses may circumvent or counter the activity of the azoles. Such an adaptive response would permit fungal survival in the presence of these antifungal agents. Indeed, in response to antifungal stress, specific signal transduction and transcriptional activation programs are affected (6). Drug efflux pump and ergosterol biosynthesis gene expression (6), the Ras-cyclic AMP (cAMP)-protein kinase A (PKA) pathway (7), the calcineurin pathway (8–10), the Ssk1p/Chk1p pathway (11), and the cell wall integrity pathway (12, 13) have all been implicated in stress responses upon azole exposure, yet the transcriptional activation pathways involved in these processes have yet to be fully elucidated. Moreover, it is likely that additional biological processes influence susceptibility to the azole antifungals as well as their ability to kill *C. albicans*.

Recently, a library of *C. albicans* transcription factor (TF) mutants was created and examined under 55 different growth conditions. Phenotypic profiling of the responses to various stress agents, including fluconazole, identified the possible biological roles of many previously uncharacterized TFs (14). While initial screens identified several TFs that affect fluconazole susceptibility, we undertook a more comprehensive analysis of this library in order to identify transcriptional activation programs that influence fluconazole killing activity and would therefore be implicated in the mechanisms by which *C. albicans* survives in the presence of the azoles. We identified one TF in particular, Cas5, whose disruption resulted in enhanced azole killing activity. This indicates a role for the Cas5 transcriptional network in the response of *C. albicans* to the azole antifungal agents.

**Received** 15 January 2014 **Returned for modification** 15 March 2014
**Accepted** 26 August 2014
**Published ahead of print** 2 September 2014
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Supplemental material for this article may be found at http://dx.doi.org/10.1128/AAC.00064-14.

doi:10.1128/AAC.00064-14

## MATERIALS AND METHODS

**Strains and growth conditions.** All *C. albicans* strains (see Table S1 in the supplemental material) were stored as frozen stocks in 40% glycerol at −80°C. YPD (1% yeast extract, 2% peptone, and 2% dextrose) agar plates and YPD liquid medium were used for routine growth of strains at 30°C (15–17). For CFU counts during time-kill analysis, PDA (0.4% potato starch, 2% dextrose, and 1.5% agar) plates were used, and cultures grown on PDA were incubated at 35°C.

**Drug susceptibility testing.** The MICs of fluconazole were determined by using broth microdilution as described by the Clinical and Laboratory Standards Institute (18, 19), modified by using both RPMI and YPD media, and were read both visually and spectrophotometrically at 24, 48, and 72 h for at least a 50% reduction in growth (20–22). Minimum fungicidal concentrations (MFCs) were measured by removing 2 μl from each well of the MIC plate and plating onto Sabouraud dextrose (SD) or YPD agar, respectively (23, 24). The MFC was defined as the lowest drug concentration that yielded no growth on plates. All MIC and MFC determinations were reproduced in triplicate. Also, serial dilutions from a suspension at an optical density at 600 nm ($OD_{600}$) of 0.1 were diluted 4-fold, and 2 μl of each dilution was plated onto YPD agar plates with and without 10 μg/ml fluconazole and then incubated at 30°C for 24 and 48 h. Fluconazole activity was also assessed by using Epsilometer test (Etest) strips (bioMérieux) according to the manufacturer's instructions, with the following modifications. Plates containing Etest strips were incubated at 30°C to mimic growth conditions utilized for MFC determinations and serial dilutions. A standardized cell suspension (0.5 McFarland standard) was used to create a confluent lawn across YPD agar plates prior to Etest strip placement and then incubated at 30°C for 24 and 48 h. Time-kill analyses were performed with a cell suspension at a 0.5 McFarland standard, which was diluted 10-fold into YPD medium with or without 10 μg/ml fluconazole and incubated at 35°C (25). Aliquots were removed at 0, 6, 12, and 24 h; 10-fold serially diluted; and plated onto PDA agar plates. CFU were counted after 48 h at 35°C, and the means and standard errors were plotted on a log-scale curve versus time. Time-kill experiments were run in triplicate.

**Screening of the TF library.** We screened a collection of strains disrupted for genes encoding transcription factors (14) in order to identify those genes required for normal susceptibility to fluconazole. We first subjected these strains to susceptibility testing for determination of both MIC by broth microdilution (18, 19) and MFC (23, 24) in RPMI medium using a 48-h endpoint and a dilution range of 0.007 to 4 μg/ml. Mutants exhibiting any reduction in MIC were then rescreened in rich YPD medium using a dilution range of 0.125 to 64 μg/ml. For this secondary screen, an earlier endpoint of 24 h was used, as by 48 h, the parent strain, SN152, was able to resume growth at the later time point at all concentrations of fluconazole tested. We then selected any mutant that exhibited a reduction in MFC in both screens for further study. Independent mutants were constructed for these transcription factor genes of interest in the SC5314 background.

**Construction of gene deletion strains.** Plasmid pBSS2 contains the entire *SAT1*-flipper disruption cassette from pSFS2 in a pBluescript vector backbone. The *SAT1*-flipper cassette consists of the *SAT1* selectable marker, which confers resistance to nourseothricin, and the *FLP* flipper recombinase gene, both flanked by FRT (flipper recombinase target) sites. The target gene's 5′-flanking sequence was cloned upstream of the *SAT1*-flipper cassette, while the 3′-flanking sequence was cloned downstream of the *SAT1*-flipper cassette (see Table S2 in the supplemental material). Upon transformation into *C. albicans* strain SC5314, the *SAT1*-flipper cassette was inserted into the coding region of one allele, and positive transformants (nourseothricin resistant [$Nou^R$]) were selected on YPD-nourseothricin agar plates containing 200 μg/ml of nourseothricin. Induction of the *FLP* gene occurred by growing the transformants in YPD medium for 24 h without selective pressure. Positive cells (nourseothricin susceptible [$Nou^S$]) were selected by replica plating onto YPD plates with or without 200 μg/ml of nourseothricin. Upon induction of the *FLP* gene, the cassette was excised such that only one copy of the *FRT* site remained in the locus. Another round was required to disrupt the second allele (26).

For gene complementation, the target gene's open reading frame (ORF) plus or minus ~1 kb was cloned in place of the target gene's 5′-flanking sequence in the above-mentioned disruption cassette. Upon transformation into strain SC5314, one allele of the gene's coding region was inserted into the original locus in conjunction with the *SAT1*-flipper cassette, and positive transformants ($Nou^R$) were selected on YPD-nourseothricin agar plates containing 200 μg/ml of nourseothricin. Induction of the *FLP* gene occurred by growing the transformants in YPD medium for 24 h without selective pressure. Positive cells ($Nou^S$) were selected by replica plating onto YPD plates with or without 200 μg/ml of nourseothricin. Upon induction of the *FLP* gene, the cassette was excised such that only the gene's allele with a downstream *FRT* site was left in the locus. Appropriate gene disruption and complementation were confirmed by Southern hybridization (26).

**Isolation of genomic DNA and Southern hybridization.** Genomic DNA was isolated as described previously (27). DNA (4 μg per sample) was digested with an appropriate restriction endonuclease, resolved on a 1% agarose gel, and, after staining with ethidium bromide, transferred by vacuum blotting onto a nylon membrane and fixed by UV cross-linking. Southern hybridization with enhanced chemiluminescence-labeled probes was performed with the Amersham ECL Direct nucleic acid labeling and detection system according to the instructions provided by the manufacturer.

**RNA isolation for quantitative reverse transcription-PCR (qRT-PCR).** RNA was isolated by using a small-scale modification of the hot-phenol method of RNA isolation described previously by Schmitt et al. (28). Briefly, cultures grown overnight were diluted to an $OD_{600}$ of 0.2 in 20 ml YPD medium and then incubated at 30°C with shaking for 3 h. Cells were collected by centrifugation, medium was decanted, and cell pellets were stored at −80°C. Cells were resuspended in 950 μl of AE buffer (50 mM sodium acetate [pH 5.3], 10 mM EDTA [pH 8.0]) and then transferred into a 2-ml RNase-free microcentrifuge tube containing 950 μl acid phenol (pH 4.3) with 1% SDS. Cells were incubated with agitation at 65°C for 10 min, and lysates were then clarified by centrifugation. The supernatant was then divided into two new 2-ml microcentrifuge tubes, each containing 950 μl of chloroform, and mixed. The sample was then subjected to centrifugation again, and the top aqueous layer was transferred into a new tube containing 1 ml of isopropanol and 100 μl 2 M sodium acetate. The RNA pellet was subsequently washed with 500 μl of 70% ethanol and collected by centrifugation. The RNA was then suspended in DNase/RNase-free $H_2O$. Quantity and purity were determined spectrophotometrically at $A_{260}$ and $A_{280}$.

**Quantitative RT-PCR.** First-strand cDNAs were synthesized from 1 μg of total RNA by using a SuperScript first-strand synthesis system for qRT-PCR (Invitrogen). Gene-specific primers (see Table S2 in the supplemental material) were designed by using Primer Express software (Applied Biosystems) and synthesized by Integrated DNA Technologies (Coralville, IA). Quantitative PCRs were performed in triplicate by using the 7000 sequence detection system (Applied Biosystems), independently amplifying *ACT1* (normalizing gene) and the genes of interest, as described previously (29). To assess statistical significance, the Student *t* test was performed by using a significance level of 0.05.

**RNA isolation for microarray.** RNA was isolated by using a large-scale version of the hot-phenol method of RNA isolation described by previously by Schmitt et al. (28). Briefly, cultures grown overnight were diluted to an $OD_{600}$ of 0.005 in 100 ml YPD medium and then incubated at 30°C with shaking for an additional 8 h to an $OD_{600}$ of 1.0. Cultures were diluted again to an $OD_{600}$ of 0.025 in 100 ml fresh YPD medium, allowed to incubate at 30°C with shaking for one doubling, inoculated with or without 10 μg/ml fluconazole, and then incubated at 30°C with shaking for 6 h. Cells were collected by centrifugation and stored at −80°C. Cell pellets were resuspended in 12 ml of AE buffer and then transferred into 50-ml Oak Ridge tubes treated with RNase Away (Molecular BioProducts) containing 12 ml acid phenol (pH 4.3) with 1% SDS.

**TABLE 1** Library hits with reduced MICs and MFCs in both RPMI (48 h) and YPD (24 h) medium[a]

| Clone | CGD name | orf19 designation | MIC (μg/ml) | | MFC (μg/ml) | |
|---|---|---|---|---|---|---|
| | | | RPMI | YPD | RPMI | YPD |
| WT | | | 0.25 | 0.5 | >4 | >64 |
| TF3 | *RPN4* | orf19.1069 | 0.0625 | 0.25 | 0.125 | 32 |
| TF33 | *CAS5* | orf19.4670 | 0.125 | 0.25 | 4 | 2 |
| TF77 | *UPC2* | orf19.391 | 0.03125 | 0.125 | 1 | 1 |
| TF104 | *CZF1* | orf19.3127 | 0.0625 | 0.5 | 0.25 | 8 |

[a] WT, wild type; CGD, Candida Genome Database.

Cells were incubated at 65°C for 10 min, and lysates were then clarified by centrifugation. The supernatant was then transferred into a new tube containing 15 ml of chloroform and mixed. The sample was then subjected to centrifugation again, and the top aqueous layer was transferred into a new tube containing 1 volume of isopropanol and 0.1 volume of 2 M sodium acetate. The RNA pellet was subsequently washed with 10 ml of 70% ethanol and collected by centrifugation. The RNA pellet was resuspended in DNase/RNase-free $H_2O$. Quantity and purity were determined spectrophotometrically at $A_{260}$ and $A_{280}$.

**Transcriptional profiling.** Gene expression profiles were obtained by hybridizing labeled cRNAs generated from *C. albicans* total RNA onto Affymetrix *C. albicans* custom expression arrays (CAN07; catalog number 49-5241) (30), which were described previously (31). Microarray hybridization and analysis were performed as described previously (31). Genes were considered to be differentially expressed in response to drug if their expression level changed by ≥1.5-fold in two independent experiments. Genes induced by fluconazole were considered to be *CAS5* dependent if induction was abrogated in the deletion mutant ≥2.0-fold (50%) less than in the wild type.

**Microarray data accession number.** All microarray data are available for download from the NCBI at the Gene Expression Omnibus website (http://www.ncbi.nlm.nih.gov/geo) under accession number GSE57930.

## RESULTS

**Disruption of Cas5 enhances fluconazole killing activity against *C. albicans*.** In order to identify transcriptional activation programs that influence the killing activity of the azole antifungals against *C. albicans*, we first screened a collection of TF deletion mutants for those mutants that displayed increased fluconazole susceptibility, as measured by broth microdilution in RPMI medium (14). In order to detect marked reductions in MICs, a smaller range of fluconazole concentrations, from 0.007 to 4 μg/ml, was used, and 19 TFs from the library with reductions in fluconazole MICs at 48 h in RPMI medium were identified. These hits were then rescreened for those TFs that also displayed reductions in fluconazole MFCs in both RPMI (48 h) and YPD (24 h) media (see Table S3 in the supplemental material). We identified four TF mutants that met the criteria: *CAS5*, *RPN4*, *UPC2*, and *CZF1* (Table 1). The observation that disruption of *UPC2* increased susceptibility to fluconazole was not surprising, as this was reported previously (20, 32, 33), validating the results of our screen. The remaining three TF deletion mutants that exhibited reductions in MFCs in both RPMI and YPD media were prioritized as being of the greatest interest, and independent mutants were generated in the SC5314 background (Table 2). In order to determine the extent to which disruption of these TF genes impacts the killing activity of fluconazole, the deletion mutants were subjected to various susceptibility tests using nutrient-rich YPD medium in order to detect strong phenotypes despite existing in an environment that promotes growth.

*CAS5* and *RPN4* disruption had a moderate impact on fluconazole MICs and a marked impact on MFCs determined by broth microdilution (Table 2) and Etest (Fig. 1A), while disruption of *CZF1* had no impact by all methods (data not shown). It should be noted that the disruption of *CAS5* resulted in slightly slower growth, which may explain the subtle changes in fluconazole susceptibility that were observed. The fluconazole MFC at 24 h in YPD for SC5314 was >64 μg/ml, whereas those for the *cas5*Δ/Δ, *rpn4*Δ/Δ, and *czf1*Δ/Δ mutants were 1, 8, and >64 μg/ml, respectively (Table 2), indicating that *CZF1* could not be independently confirmed as influencing susceptibility to fluconazole, and therefore, *CZF1* did not proceed through any further examination. For SC5314, the 48-h MIC determined by Etest was 1.0 μg/ml, and a halo of reduced growth was observed up to the Etest strip, consistent with the fungistatic nature of fluconazole. At 48 h, the MICs determined by Etest for the *cas5*Δ/Δ and *rpn4*Δ/Δ mutants were 0.25 μg/ml and 1.5 μg/ml, respectively. A large clear zone of inhibition was observed for the *cas5*Δ/Δ mutant, but the zone of inhibition was smaller for the *rpn4*Δ/Δ mutant (Fig. 1A). Similar changes in fluconazole MICs, MFCs, and zones of inhibition determined by Etest were likewise observed for the previously reported *cas5*Δ/Δ mutant strain 1186 compared to its parent strain BWP17 (data not shown) (34). Using a 72-h endpoint for a broth microdilution assay using YPD medium as a way to assess the requirement of *CAS5* for growth in the presence of fluconazole, we found that SC5314 and the *rpn4*Δ/Δ mutant were able to resume growth at all concentrations of fluconazole tested, whereas the *cas5*Δ/Δ mutant grew only at the lowest concentrations (Fig. 1B). When cells were plated onto YPD agar plates containing 10 μg/ml of fluconazole, growth was reduced for the *cas5*Δ/Δ mutant but only slightly for the *rpn4*Δ/Δ mutant compared to SC5314 (Fig. 1C). Time-kill analysis showed enhanced killing activity for 10 μg/ml fluconazole against the *cas5*Δ/Δ mutant but not the *rpn4*Δ/Δ mutant compared to the parent strain (Fig. 1D). *RPN4*

**TABLE 2** MICs and MFCs in YPD medium in the SC5314 background

| Strain | Relevant genotype[a] | MIC (μg/ml) | | | MFC (μg/ml) | | |
|---|---|---|---|---|---|---|---|
| | | 24 h | 48 h | 72 h | 24 h | 48 h | 72 h |
| SC5314 | *CAS5*/*CAS5* | 0.5 | 0.5 | >64 | >64 | >64 | >64 |
| *cas5*Δ/Δ | *cas5*Δ::FRT/*cas5*Δ::FRT | 0.5 | 1 | 1 | 1 | 1 | 1 |
| *cas5*Δ/Δ+*CAS5* | *cas5*Δ::FRT/*CAS5*-ca*SAT1* | 0.5 | 0.5 | >64 | >64 | >64 | >64 |
| *rpn4*Δ/Δ | *rpn4*Δ::FRT/*rpn4*Δ::FRT | 0.5 | 1 | >64 | 8 | 8 | 8 |
| *rpn4*Δ/Δ+*RPN4* | *rpn4*Δ::FRT/*RPN4*-ca*SAT1* | 0.5 | 0.5 | >64 | >64 | >64 | >64 |
| *czf1*Δ/Δ | *czf1*Δ::FRT/*czf1*Δ::FRT | 0.5 | 0.5 | >64 | >64 | >64 | >64 |

[a] ca*SAT1*, *C. albicans SAT1*.

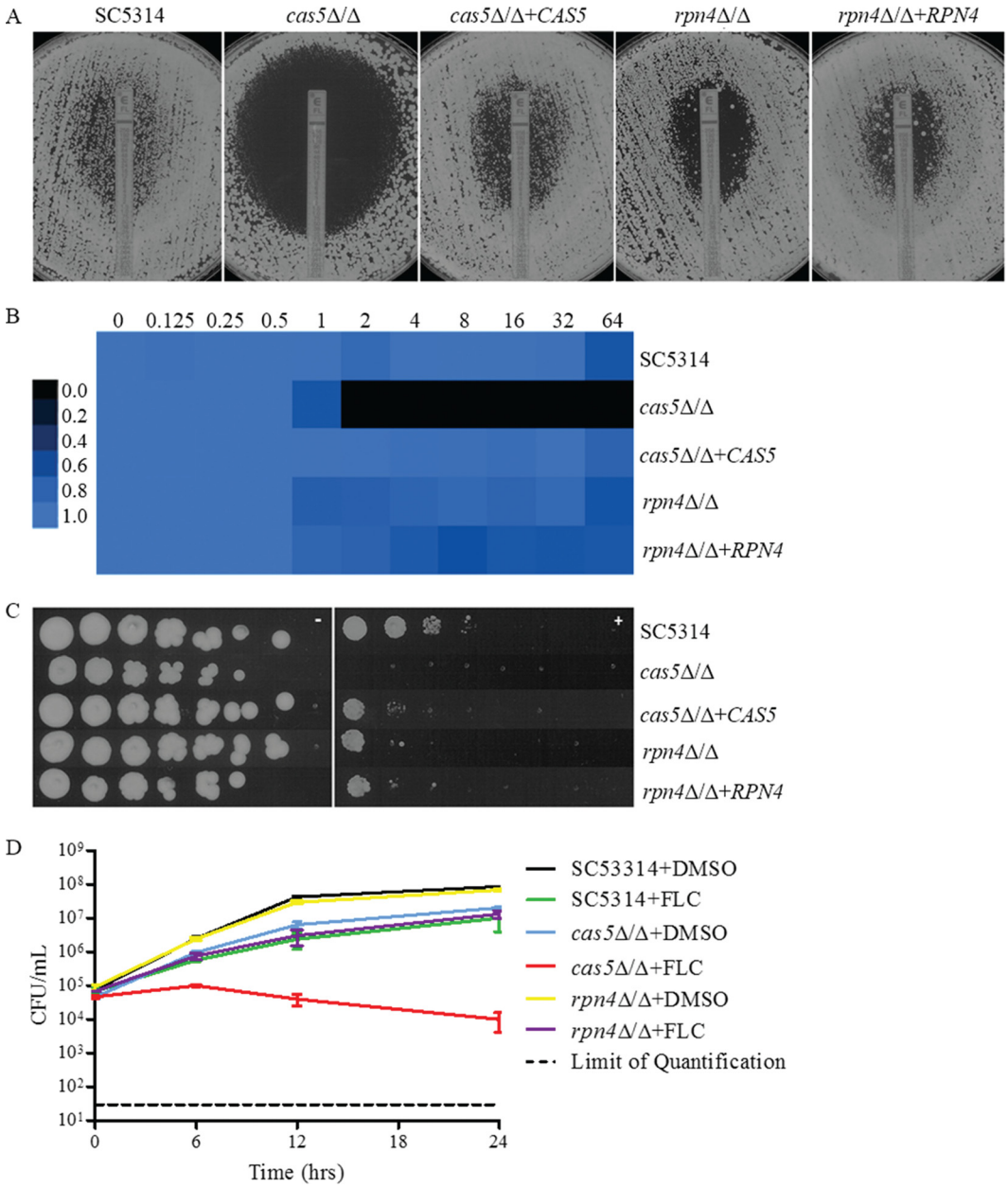


**FIG 1** (A) Effect of *CAS5* and *RPN4* on MIC and growth on YPD agar as determined by Etest. A confluent lawn of *C. albicans* was streaked prior to the addition of Etest strips and then incubated for 48 h. (B) MIC heat map of SC5314, mutants, and complemented derivatives. Susceptibility was determined by broth microdilution in YPD medium at 72 h. Growth was quantified spectrophotometrically and assigned to a colorimetric scale. (C) Effect of *CAS5* and *RPN4* on growth on solid medium containing fluconazole. From 4-fold serial dilutions of *C. albicans* strains, 2-μl aliquots were spotted onto YPD agar with or without 10 μg/ml FLC and incubated for 48 h. (D) Effect of fluconazole on *CAS5* and *RPN4* in time-kill assays. SC5314, *cas5*Δ/Δ, or *rpn4*Δ/Δ cells were diluted in YPD medium containing FLC (10 μg/ml) or the solvent dimethyl sulfoxide (DMSO). After 0, 6, 12, and 24 h, samples from each dilution were diluted and plated for CFU. Shown are the means of data from three independent experiments with standard error bars.

was not pursued in further analyses due to its minimal impact at the therapeutically relevant concentration of fluconazole, 10 μg/ml. All phenotypes were reverted by reintegration of one allele of the disrupted gene.

**Disruption of Cas5 does not override combinations of clinical drug resistance mechanisms.** In order to determine whether Cas5 influences the fluconazole susceptibility of azole-resistant clinical isolates, we constructed *cas5*Δ/Δ mutant strains in the background of an azole-resistant clinical isolate (12-99) known to constitutively maintain four of the most common mechanisms of azole resistance: overexpression of *CDR1* and *CDR2*, overexpression of *MDR1*, overexpression of *ERG11*, and mutation in *ERG11* (35). The disruption of *CAS5* in isolate 12-99 (12-99*cas5*Δ/Δ) did not result in a reduction in the MIC or MFC and therefore was not able to overcome the combined mechanisms of resistance operative in this isolate (Table 3). Time-kill analysis revealed an overall growth defect, but this did not alter the effect of fluconazole against 12-99*cas5*Δ/Δ at 10 μg/ml (Fig. 2).

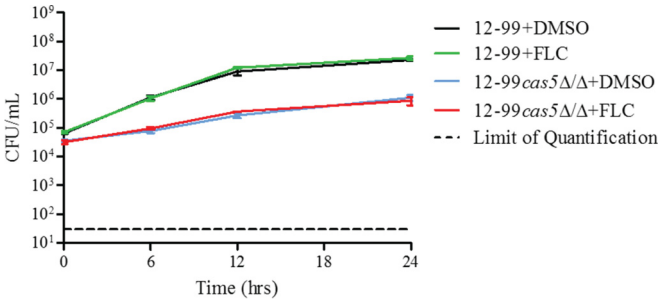


**FIG 2** Effect of fluconazole on *CAS5* in 12-99 as determined by time-kill assays. 12-99 or 12-99*cas5*Δ/Δ cells were diluted in YPD medium containing FLC (10 μg/ml) or the solvent dimethyl sulfoxide (DMSO). After 0, 6, 12, and 24 h, samples from each dilution were diluted and plated for CFU. Shown are the means of data from three independent experiments with standard error bars.

Isolate 12-99 simultaneously overexpresses *CDR1* and *CDR2* as well as *MDR1* (36). Since previous studies have shown that azole-resistant isolates usually do not simultaneously overexpress these transporter genes (37–39), *CAS5* was also disrupted in clinical isolates S2, G5, and Gu5. Isolate S2 was previously shown to have a G-to-S amino acid substitution at position 464 (G464S) in Upc2p, which renders it constitutively active, resulting in the upregulation of *ERG11* and increased fluconazole resistance (30, 40). Isolate G5 has a G997V amino acid substitution in Mrr1p, resulting in increased *MDR1* expression levels, and is also homozygous for an *ERG11* mutation (40, 41). Isolate Gu5 displays increased expression levels of *CDR1* and *CDR2* (42). As with isolate 12-99, the disruption of *CAS5* in these three clinical isolates did not markedly alter the MIC or MFC in these resistant backgrounds when examined by broth microdilution (Table 3).

**Disruption of *CAS5* in strains containing resistance mutations in *MRR1*, *TAC1*, and *ERG11* impacts fluconazole susceptibility.** Since the deletion of *CAS5* was unable to override common resistance mechanisms acting together, we sought to investigate the requirement for *CAS5* for specific mechanisms of resistance. Independent mutants were constructed in strains containing two copies of a gene conferring reduced susceptibility to fluconazole: *MRR1* encoding a P683S mutation (*MRR1*$^{P683S}$), *TAC1*$^{G980E}$, *UPC2*$^{G648D}$, or *ERG11*$^{K143R}$. The *MRR1*$^{P683S}$, *TAC1*$^{G980E}$, and *UPC2*$^{G648D}$ alleles contain gain-of-function (GOF) mutations which render the transcription factors that they encode constitutively active, resulting in the upregulation of *MDR1*, *CDR1* and *CDR2*, and *ERG11*, respectively, and decreased fluconazole susceptibility (30, 40, 43–45). The *ERG11*$^{K143R}$ allele contains a point mutation postulated to be located near the azole access channel, interfering with the entry of fluconazole, which results in decreased fluconazole susceptibility (46). Interestingly, we observed that *UPC2*$^{G648D}$ *cas5*Δ/Δ mutant cells were larger than those of the parent strain and also were elongated and clumped together. As a result, susceptibility testing results by both broth microdilution and Etest for this strain were inconsistent due to the extreme variability in optical density versus cell density and therefore were not reported. The disruption of *CAS5* in each remaining background did not markedly impact the MICs at 24 h but did result in reductions in MICs at later time points by all methods. The MFCs at 48 h in YPD medium for *ERG11*$^{K143R}$ *cas52*Δ/Δ, *MRR1*$^{P683S}$ *cas5*Δ/Δ, and *TAC1*$^{G980E}$ *cas5*Δ/Δ mutants were all reduced from 8 μg/ml, >64 μg/ml, and >64 μg/ml in their background strains to 4 μg/ml, 8 μg/ml, and 16 μg/ml, respectively (Table 4). At 48 h, the MICs for *ERG11*$^{K143R}$ *cas52*Δ/Δ, *MRR1*$^{P683S}$ *cas5*Δ/Δ, and *TAC1*$^{G980E}$ *cas5*Δ/Δ mutants determined by Etest were all reduced from 1.5 μg/ml, 6 μg/ml, and 8 μg/ml in their background strains to 0.5 μg/ml, 1.5 μg/ml, and 6 μg/ml, respectively (Fig. 3A). When cells were plated onto YPD agar plates containing 10 μg/ml fluconazole, growth was slightly reduced in the presence of fluconazole for the *cas5*Δ/Δ mutants compared to their respective backgrounds (Fig. 3B).

**Expression of *ERG11*, *CDR1*, *CDR2*, and *MDR1* when *CAS5* is disrupted in resistant backgrounds.** One possible explanation for the enhanced killing activity of fluconazole in the absence of Cas5 could be decreased expression levels of *ERG11* or genes encoding the Cdr1 and Cdr2 or Mdr1 efflux pump. In order to determine if this was the case, we measured *ERG11*, *CDR1*, *CDR2*, and *MDR1* mRNA abundances by qRT-PCR in strains containing a single resistance mechanism and their respective *cas5*Δ/Δ mutants (Fig. 4). Interestingly, the *cas5*Δ/Δ mutant constructed in the SC5314 background showed a slight reduction in the baseline *ERG11* expression level. This was also the case for the *cas5*Δ/Δ mutants constructed in the *MRR1*$^{P683S}$ and *UPC2*$^{G648D}$ backgrounds. Disruption of *CAS5* did not result in decreased *CDR1*

**TABLE 3** MICs and MFCs in YPD medium in the background of resistant clinical strains

| Strain | Relevant characteristic or genotype | MIC (μg/ml) | | | MFC (μg/ml) | | |
|---|---|---|---|---|---|---|---|
| | | 24 h | 48 h | 72 h | 24 h | 48 h | 72 h |
| SC5314 | *CAS5*/*CAS5* | 0.5 | 0.5 | >64 | >64 | >64 | >64 |
| 2-79 | Susceptible isolate | 1 | 1 | >64 | >64 | >64 | >64 |
| 12-99 | Resistant isolate | >64 | >64 | >64 | >64 | >64 | >64 |
| 12-99*cas5*Δ/Δ | *cas5*Δ::FRT/*cas5*Δ::FRT | >64 | >64 | >64 | >64 | >64 | >64 |
| G2 | Susceptible isolate | 1 | >64 | >64 | >64 | >64 | >64 |
| G5 | Resistant isolate | >64 | >64 | >64 | >64 | >64 | >64 |
| G5*cas5*Δ/Δ | *cas5*Δ::FRT/*cas5*Δ::FRT | 32 | 32 | 32 | >64 | >64 | >64 |
| Gu2 | Susceptible isolate | 0.5 | 1 | >64 | >64 | >64 | >64 |
| Gu5 | Resistant isolate | >64 | >64 | >64 | >64 | >64 | >64 |
| Gu5*cas5*Δ/Δ | *cas5*Δ::FRT/*cas5*Δ::FRT | >64 | >64 | >64 | >64 | >64 | >64 |
| S1 | Susceptible isolate | 2 | >64 | >64 | >64 | >64 | >64 |
| S2 | Resistant isolate | >64 | >64 | >64 | >64 | >64 | >64 |
| S2*cas5*Δ/Δ | *cas5*Δ::FRT/*cas5*Δ::FRT | 64 | >64 | >64 | >64 | >64 | >64 |

**TABLE 4** MICs and MFCs in YPD in the background of strains expressing resistance mechanisms

| Strain | Relevant characteristic or genotype | MIC (μg/ml) | | | MFC (μg/ml) | | |
|---|---|---|---|---|---|---|---|
| | | 24 h | 48 h | 72 h | 24 h | 48 h | 72 h |
| SC5314 | *CAS5*/*CAS5* | 0.5 | 0.5 | >64 | >64 | >64 | >64 |
| *ERG11*$^{K143R}$ | *ERG11*$^{K143R}$::FRT/*ERG11*$^{K143R}$::FRT *CAS5*/*CAS5* | 4 | 8 | 8 | 4 | 8 | 8 |
| *ERG11*$^{K143R}$ *cas5*Δ/Δ | *ERG11*$^{K143R}$::FRT/*ERG11*$^{K143R}$::FRT *cas5*Δ::FRT/*cas5*Δ::FRT | 4 | 4 | 4 | 4 | 4 | 4 |
| *MRR1*$^{P683S}$ | *MRR1*$^{P683S}$::FRT/*MRR1*$^{P683S}$::FRT *CAS5*/*CAS5* | 8 | 16 | >64 | >64 | >64 | >64 |
| *MRR1*$^{P683S}$ *cas5*Δ/Δ | *MRR1*$^{P683S}$::FRT/*MRR1*$^{P683S}$::FRT *cas5*Δ::FRT/*cas5*Δ::FRT | 8 | 8 | 8 | 8 | 8 | 8 |
| *TAC1*$^{G980E}$ | *TAC1*$^{G980E}$::FRT/*TAC1*$^{G980E}$::FRT *CAS5*/*CAS5* | 8 | 16 | >64 | >64 | >64 | >64 |
| *TAC1*$^{G980E}$ *cas5*Δ/Δ | *TAC1*$^{G980E}$::FRT/*TAC1*$^{G980E}$::FRT *cas5*Δ::FRT/*cas5*Δ::FRT | 8 | 16 | 16 | 16 | 16 | 16 |

expression levels. Alternatively, disruption of *CAS5* increased *CDR2* expression levels in all backgrounds, except for *TAC1*$^{G980E}$ and *MRR1*$^{P683S}$. Likewise, *MDR1* expression levels also increased with the disruption of *TAC1* in the SC5314 and *TAC1*$^{G980E}$ backgrounds. The significance of the increased expression levels of these transporter genes is unclear. These data suggest that the enhanced killing activity of fluconazole observed for resistant strains lacking *CAS5* is not due to decreased levels of transporter gene

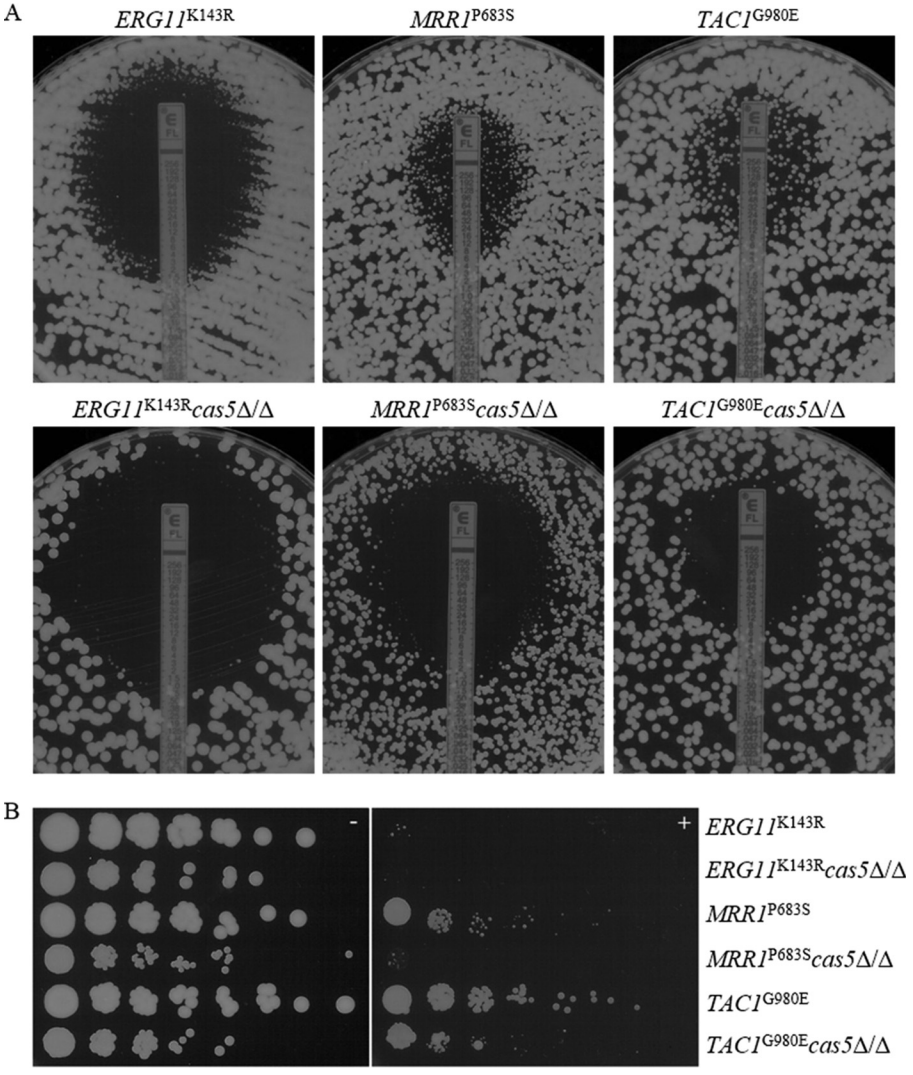


**FIG 3** (A) Effect of *CAS5* in isogenic resistant backgrounds on MIC and growth on YPD agar as determined by Etest. A confluent lawn of *C. albicans* was streaked prior to the addition of Etest strips and then incubated for 48 h. (B) Effect of *CAS5* on the ability of isogenic strains to grow on solid medium containing fluconazole. From 4-fold serial dilutions of *C. albicans* strains, 2-μl aliquots were spotted onto YPD agar with or without 10 μg/ml FLC and incubated for 48 h.

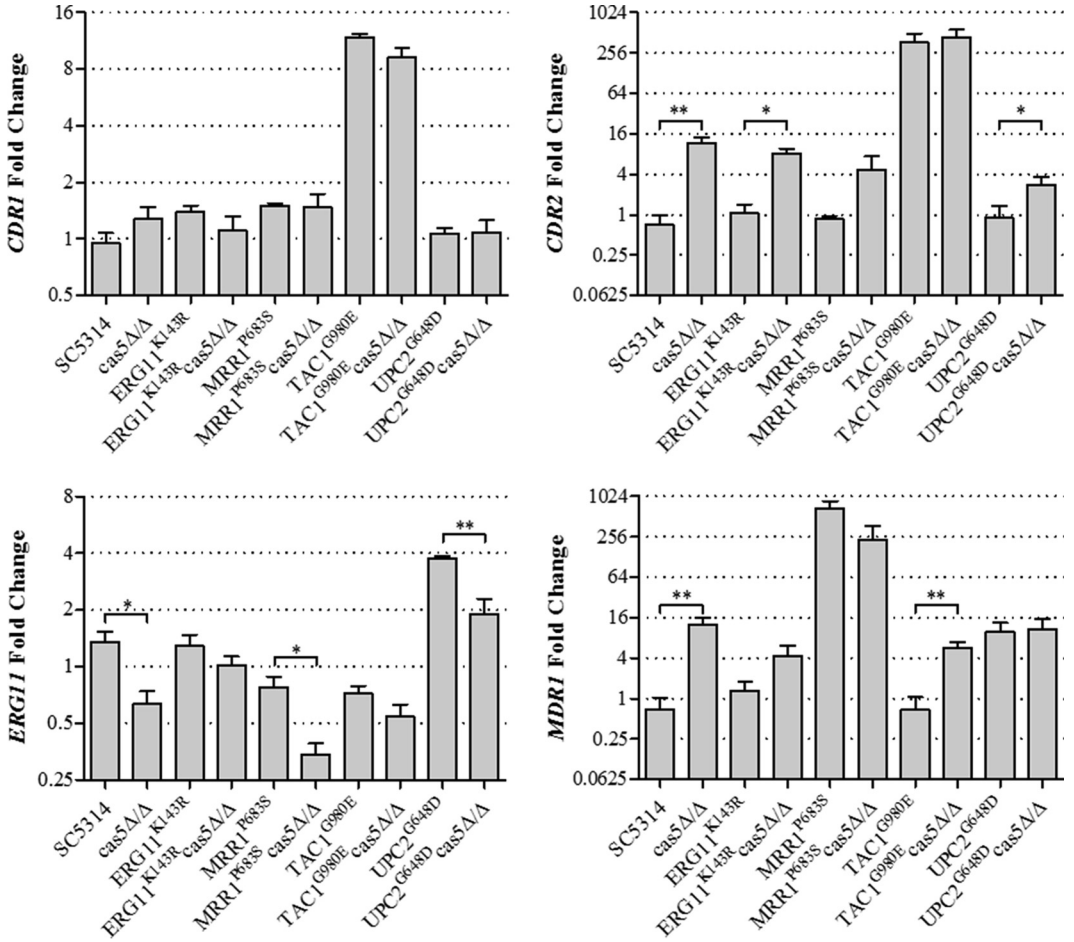


**FIG 4** Expression levels of *ERG11*, *CDR1*, *CDR2*, and *MDR1* in mutants compared to those in SC5314. Expression levels of *ERG11*, *CDR1*, *CDR2*, and *MDR1* were measured in triplicate by qRT-PCR and compared to the expression levels in SC5314. Error bars represent the standard errors of the means. Asterisks represent significance levels (*, $P < 0.05$; **, $P < 0.01$).

expression. Instead, the observed phenotypes may be associated with reductions in *ERG* gene expression levels, particularly *ERG11*.

**Comparison of the gene expression profiles of wild-type strain SC5314 and the *cas5*Δ/Δ mutant exposed to fluconazole.** In order to identify genes whose expression in response to fluconazole is influenced by Cas5, we compared the transcriptional profiles of SC5314 and its *cas5*Δ/Δ derivative after treatment with or without 10 μg/ml fluconazole for 6 h. Genes were considered to be differentially expressed in response to fluconazole if their expression levels changed by ≥1.5-fold in two independent experiments (see Dataset S1 in the supplemental material). Fluconazole-inducible genes were also considered to be *CAS5* dependent if their induction was abrogated in the deletion mutant and was ≥2.0-fold (50%) less than that of SC5314. Using these criteria, there were 209 genes upregulated by fluconazole whose induction was abrogated in the absence of *CAS5* (Table 5). The most common biological processes represented by these genes include transport, response to chemical stimulus, the oxidation-reduction process, filamentous growth, the lipid metabolic process, and cell wall organization. Interestingly, there were also six genes involved in iron ion transport, binding, and homeostasis (*CFL2*, *CFL4*, *FET34*, *FRE10*, *FTR1*, and orf19.1411).

**Validation of microarray data by real-time RT-PCR.** In order to validate the differential expression of genes identified by microarray, we examined the mRNA abundances of five genes of interest using the same RNA isolated for the microarray experiments. *PGA13* was chosen because it is involved in cell wall organization and was also identified previously by Bruno et al. to be Cas5 dependent (34). In addition, four other genes were chosen based on their involvement in cell wall organization (*PGA31*), iron ion transport (*CFL4* and *FTR1*), and the lipid metabolic process (*ERG26*). The microarray data and the real-time RT-PCR results were consistent (Fig. 5). The expressions of the *CFL4*, *ERG26*, *FTR1*, *PGA13*, and *PGA31* genes were upregulated in wild-type strain SC5314 when treated with fluconazole but could not respond to the same extent when *CAS5* was disrupted.

## DISCUSSION

Identifying transcriptional networks that are central to azole antifungal killing activity would be instructive for developing cotherapeutic strategies that could enhance their activity against *C. albicans*. Several signal transduction and transcriptional activation programs have been identified to respond to antifungal stress, but the transcription factors and their respective target genes have

**TABLE 5** Genes upregulated by ≥1.5-fold by fluconazole that are dependent upon Cas5

| Process[a] | orf19 designation | CGD | Fold change in expression: SC5314 + FLC/ SC5314 (A) Expt 1 | SC5314 + FLC/ SC5314 (A) Expt 2 | *cas5*Δ/Δ+ FLC/ *cas5*Δ/Δ (B) Expt 1 | *cas5*Δ/Δ+ FLC/ *cas5*Δ/Δ (B) Expt 2 | B/A ratio Expt 1 | B/A ratio Expt 2 |
|---|---|---|---|---|---|---|---|---|
| Lipid metabolic process | orf19.10 | *ALK8* | 9.0 | 8.1 | 3.0 | 3.5 | 0.3 | 0.4 |
| | orf19.2248 | *ARE2* | 1.7 | 1.6 | 0.7 | 0.7 | 0.4 | 0.4 |
| | orf19.2670 | | 1.6 | 1.8 | 0.4 | 0.4 | 0.3 | 0.2 |
| | orf19.2909 | *ERG26* | 2.4 | 1.7 | 0.9 | 0.9 | 0.4 | 0.5 |
| | orf19.4897 | *SFH5* | 2.4 | 2.4 | 1.3 | 1.2 | 0.5 | 0.5 |
| | orf19.4982 | | 2.1 | 2.3 | 0.9 | 0.6 | 0.4 | 0.3 |
| | orf19.5640 | *PEX5* | 2.2 | 2.6 | 0.9 | 0.9 | 0.4 | 0.3 |
| | orf19.5751 | *ORM1* | 3.3 | 3.6 | 1.6 | 1.7 | 0.5 | 0.5 |
| | orf19.6594 | *PLB3* | 2.1 | 3.4 | 1.1 | 0.9 | 0.5 | 0.3 |
| Cell wall organization | orf19.212 | *VPS28* | 1.7 | 2.1 | 0.5 | 0.5 | 0.3 | 0.3 |
| | orf19.5302 | *PGA31* | 17.6 | 4.1 | 2.1 | 1.5 | 0.1 | 0.4 |
| | orf19.5644 | | 2.3 | 3.5 | 1.1 | 1.0 | 0.5 | 0.3 |
| | orf19.6102 | *RCA1* | 2.5 | 3.4 | 1.2 | 1.2 | 0.5 | 0.4 |
| | orf19.6420 | *PGA13* | 15.6 | 30.6 | 2.4 | 2.3 | 0.2 | 0.1 |
| | orf19.6481 | *YPS7* | 1.6 | 2.3 | 0.4 | 0.4 | 0.3 | 0.2 |
| | orf19.719 | | 1.8 | 3.3 | 1.0 | 1.0 | 0.5 | 0.3 |
| Iron ion transport | orf19.1415 | *FRE10* | 2.0 | 4.2 | 0.9 | 2.3 | 0.5 | 0.5 |
| | orf19.1932 | *CFL4* | 35.6 | 359 | 2.4 | 6.6 | 0.1 | 0.0 |
| | orf19.4215 | *FET34* | 2.1 | 5.2 | 0.2 | 0.5 | 0.1 | 0.1 |
| | orf19.7219 | *FTR1* | 3.2 | 7.8 | 0.5 | 1.1 | 0.1 | 0.1 |
| Iron ion binding | orf19.1411 | | 2.7 | 4.8 | 1.1 | 0.9 | 0.4 | 0.2 |
| Iron ion homeostasis | orf19.1264 | *CFL2* | 2.0 | 6.0 | 0.6 | 0.5 | 0.3 | 0.1 |
| Transport | orf19.111 | *CAN2* | 1.7 | 13.0 | 0.7 | 1.5 | 0.4 | 0.1 |
| | orf19.1252 | *YME1* | 1.9 | 3.0 | 1.0 | 0.7 | 0.5 | 0.2 |
| | orf19.1313 | *CDR3* | 1.9 | 2.1 | 0.9 | 1.0 | 0.5 | 0.5 |
| | orf19.1352 | *TIM22* | 3.6 | 4.4 | 0.9 | 1.1 | 0.2 | 0.3 |
| | orf19.1563 | *ECM3* | 1.5 | 1.5 | 0.4 | 0.6 | 0.3 | 0.4 |
| | orf19.1867 | | 2.6 | 4.2 | 0.7 | 0.6 | 0.3 | 0.2 |
| | orf19.2073 | | 1.5 | 1.6 | 0.6 | 0.5 | 0.4 | 0.3 |
| | orf19.2292 | *OPT4* | 5.2 | 3.7 | 1.2 | 1.0 | 0.2 | 0.3 |
| | orf19.2350 | | 2.5 | 4.1 | 0.6 | 1.0 | 0.3 | 0.3 |
| | orf19.2810 | *AAP1* | 18.9 | 19.6 | 7.5 | 8.8 | 0.4 | 0.4 |
| | orf19.2946 | *HNM4* | 8.4 | 4.9 | 2.9 | 2.5 | 0.3 | 0.5 |
| | orf19.3015 | *ARX1* | 2.7 | 3.9 | 1.3 | 1.7 | 0.5 | 0.4 |
| | orf19.3195 | *HIP1* | 1.5 | 2.6 | 0.6 | 0.7 | 0.4 | 0.3 |
| | orf19.3232 | | 24.6 | 5.3 | 2.6 | 1.0 | 0.1 | 0.2 |
| | orf19.3574 | *MDJ2* | 5.6 | 3.1 | 2.0 | 1.2 | 0.4 | 0.4 |
| | orf19.4041 | *PEX4* | 4.9 | 4.3 | 1.3 | 1.5 | 0.3 | 0.4 |
| | orf19.4090 | | 2.1 | 1.7 | 1.1 | 0.8 | 0.5 | 0.5 |
| | orf19.4372 | | 1.8 | 3.0 | 0.6 | 0.6 | 0.3 | 0.2 |
| | orf19.4384 | *HXT5* | 70.6 | 75.5 | 11.1 | 23.4 | 0.2 | 0.3 |
| | orf19.4546 | *HOL4* | 4.3 | 5.1 | 1.7 | 2.3 | 0.4 | 0.4 |
| | orf19.4682 | *HGT17* | 45.0 | 24.7 | 7.0 | 7.3 | 0.2 | 0.3 |
| | orf19.4887 | *ECM21* | 2.7 | 3.1 | 0.8 | 1.0 | 0.3 | 0.3 |
| | orf19.5539 | | 1.6 | 1.6 | 0.7 | 0.8 | 0.5 | 0.5 |
| | orf19.5902 | *RAS2* | 7.6 | 6.2 | 0.8 | 1.1 | 0.1 | 0.2 |
| | orf19.5958 | *CDR2* | 2.1 | 2.5 | 0.9 | 1.0 | 0.4 | 0.4 |
| | orf19.6117 | | 1.5 | 2.3 | 0.6 | 0.4 | 0.4 | 0.2 |
| | orf19.6249 | *HAK1* | 4.4 | 10.7 | 2.1 | 3.2 | 0.5 | 0.3 |
| | orf19.6648 | *SDA1* | 3.2 | 6.8 | 1.6 | 3.2 | 0.5 | 0.4 |
| | orf19.6993 | *GAP2* | 21.7 | 8.6 | 3.0 | 4.5 | 0.1 | 0.5 |
| | orf19.7056 | | 6.2 | 120.3 | 2.5 | 2.7 | 0.4 | 0.0 |
| | orf19.7093 | *HGT13* | 30.7 | 13.2 | 2.0 | 1.5 | 0.1 | 0.1 |
| | orf19.7094 | *HGT12* | 423.2 | 19.8 | 8.0 | 1.9 | 0.0 | 0.1 |

(Continued on following page)

**TABLE 5** (Continued)

| Process[a] | orf19 designation | CGD | Fold change in expression: SC5314 + FLC/ SC5314 (A) Expt 1 | SC5314 + FLC/ SC5314 (A) Expt 2 | *cas5*Δ/Δ+ FLC/ *cas5*Δ/Δ (B) Expt 1 | *cas5*Δ/Δ+ FLC/ *cas5*Δ/Δ (B) Expt 2 | B/A ratio Expt 1 | B/A ratio Expt 2 |
|---|---|---|---|---|---|---|---|---|
| Response to chemical stimulus | orf19.2060 | *SOD5* | 33.2 | 14.2 | 5.4 | 6.1 | 0.2 | 0.4 |
| | orf19.2838 | | 2.5 | 2.9 | 0.8 | 0.7 | 0.3 | 0.3 |
| | orf19.2876 | *CBF1* | 2.3 | 3.2 | 0.5 | 0.5 | 0.2 | 0.2 |
| | orf19.3159 | *UTP20* | 3.8 | 8.9 | 1.9 | 3.5 | 0.5 | 0.4 |
| | orf19.3736 | *KAR4* | 2.8 | 2.6 | 1.2 | 1.0 | 0.4 | 0.4 |
| | orf19.4015 | *CAG1* | 3.1 | 3.6 | 0.8 | 0.7 | 0.3 | 0.2 |
| | orf19.4317 | *GRE3* | 1.5 | 1.5 | 0.8 | 0.6 | 0.5 | 0.4 |
| | orf19.4318 | *MIG1* | 1.7 | 2.1 | 0.7 | 1.0 | 0.4 | 0.5 |
| | orf19.5326 | | 1.5 | 1.9 | 0.6 | 0.7 | 0.4 | 0.4 |
| | orf19.5591 | *ADO1* | 2.4 | 2.9 | 01.1 | 1.5 | 0.4 | 0.5 |
| | orf19.6202 | *RBT4* | 3.3 | 6.2 | 1.6 | 2.2 | 0.5 | 0.3 |
| | orf19.6881 | *YTH1* | 1.9 | 2.0 | 0.9 | 0.9 | 0.4 | 0.4 |
| | orf19.7316 | | 2.0 | 2.3 | 0.7 | 0.8 | 0.3 | 0.3 |
| | orf19.7384 | *NOG1* | 3.7 | 7.2 | 1.2 | 2.1 | 0.3 | 0.3 |
| Filamentous growth | orf19.2397.3 | | 2.0 | 4.3 | 0.9 | 1.2 | 0.5 | 0.3 |
| | orf19.4055 | | 1.9 | 2.5 | 0.9 | 0.7 | 0.5 | 0.3 |
| | orf19.4815 | *YTM1* | 2.3 | 3.2 | 0.6 | 1.4 | 0.3 | 0.4 |
| | orf19.4928 | *SEC2* | 2.0 | 2.1 | 0.4 | 0.4 | 0.2 | 0.2 |
| | orf19.5741 | *ALS1* | 66.8 | 79.0 | 22.6 | 21.0 | 0.3 | 0.3 |
| | orf19.5798 | *LIG4* | 1.8 | 2.6 | 0.9 | 0.8 | 0.5 | 0.3 |
| | orf19.6595 | *RTA4* | 10.4 | 15.0 | 3.0 | 2.9 | 0.3 | 0.2 |
| | orf19.6888 | | 4.3 | 5.7 | 1.4 | 1.1 | 0.3 | 0.2 |
| | orf19.7313 | *SSU1* | 4.5 | 4.8 | 1.3 | 1.1 | 0.3 | 0.2 |
| | orf19.7374 | *CTA4* | 1.7 | 2.2 | 0.8 | 1.1 | 0.5 | 0.5 |
| | orf19.7436 | *AAF1* | 2.0 | 4.4 | 1.0 | 1.2 | 0.5 | 0.3 |
| | orf19.795 | *VPS36* | 2.0 | 2.5 | 0.6 | 0.6 | 0.3 | 0.3 |
| Oxidation-reduction process | orf19.1117 | | 5.7 | 2.3 | 0.7 | 1.1 | 0.1 | 0.5 |
| | orf19.1473 | | 35.0 | 15.0 | 3.8 | 4.8 | 0.1 | 0.3 |
| | orf19.2108 | *SOD6* | 7.9 | 10.6 | 1.6 | 1.1 | 0.2 | 0.1 |
| | orf19.3538 | *FRE9* | 4.7 | 14.9 | 2.5 | 3.6 | 0.5 | 0.2 |
| | orf19.3707 | *YHB1* | 3.0 | 2.8 | 0.9 | 0.6 | 0.3 | 0.2 |
| | orf19.4747 | *HEM14* | 1.7 | 2.0 | 0.6 | 0.4 | 0.3 | 0.2 |
| | orf19.4871 | *ERO1* | 1.9 | 1.9 | 0.9 | 0.6 | 0.5 | 0.3 |
| | orf19.5879 | | 3.3 | 2.9 | 1.5 | 1.2 | 0.5 | 0.4 |
| | orf19.638 | *FDH1* | 22.7 | 19.5 | 4.4 | 3.9 | 0.4 | 0.2 |
| | orf19.6837 | *FMA1* | 1.5 | 1.5 | 0.7 | 0.7 | 0.5 | 0.4 |
| | orf19.7111.1 | *SOD3* | 40.5 | 69.2 | 9.4 | 15.0 | 0.2 | 0.2 |
| | orf19.7314 | *CDG1* | 7.5 | 6.0 | 1.0 | 0.6 | 0.1 | 0.1 |
| | orf19.742 | *ALD6* | 11.5 | 10.4 | 4.1 | 1.7 | 0.4 | 0.2 |
| | orf19.7551 | *ALO1* | 1.5 | 2.8 | 0.7 | 0.6 | 0.5 | 0.2 |

[a] Descriptions are from the Candida Genome Database (http://www.candidagenome.org/).

yet to be identified for many of these processes (6–13). In the present study, we screened a library of *C. albicans* mutants disrupted for genes encoding TFs in order to identify those genes which resulted in increased killing activity of fluconazole. We observed that disruption of *CAS5* resulted in slightly reduced MICs but also resulted in substantial reductions in fluconazole MFCs at 24, 48, and 72 h. Indeed, disruption of *CAS5* in an azole-susceptible strain prevented its regrowth in YPD medium in the presence of higher fluconazole concentrations after 72 h, resulted in a clear zone of inhibition around a fluconazole Etest strip, and prevented growth on solid medium containing a therapeutically relevant concentration of fluconazole (10 μg/ml). Time-kill analysis also demonstrated an enhanced killing effect of 10 μg/ml fluconazole against the *cas5*Δ/Δ mutant compared to its parent strain. The disruption of *RPN4* did not display as strong of an effect, suggesting that *RPN4* influences fungistatic azole activity to a lesser extent, while *CZF1* disruption had no impact on the killing activity of fluconazole. As previously observed for disruption of *UPC2*, *CAS5* disruption enhanced the killing activity of fluconazole (20).

Upon disruption of *CAS5* in fluconazole-resistant clinical isolates 12-99, G5, Gu5, and S2, which carry combinations of the four most common mechanisms of resistance, there was no marked change in fluconazole killing activity, although there was a slight change in susceptibility in the G5 background. The disruption of *CAS5* in isogenic strains containing specific resistance mutations in *ERG11*, *MRR1*, or *TAC1* resulted in slightly increased killing

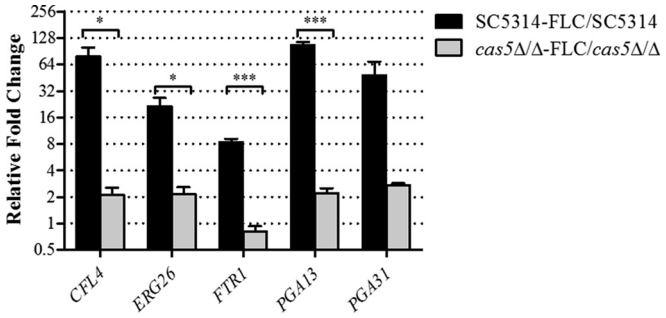


**FIG 5** Validation of fluconazole-inducible and Cas5-dependent gene expression. Expression levels of *CFL4*, *ERG26*, *FTR1*, *PGA13*, and *PGA31* were measured in triplicate by qRT-PCR and compared to the expression levels in SC5314. Shown are the relative *n*-fold changes in gene expression in SC5314 and *cas5*Δ/Δ mutants treated with fluconazole (FLC). Error bars represent the standard errors of the means. Asterisks represent significance levels (*, $P < 0.05$; ***, $P < 0.001$).

activity. However, the strain containing the *UPC2*$^{G648D}$ GOF mutation rendered irregular cell shape and size. This phenotype was also present when *CAS5* was disrupted in strains constructed to contain a *UPC2*$^{G648S}$ or *UPC2*$^{A643V}$ allele (data not shown). This may suggest a relationship between the two TFs. When *UPC2* is constitutively active, ergosterol biosynthesis genes are upregulated, and cellular ergosterol content increases (29), consequently impacting membrane fluidity, which affects cell wall composition (47). *CAS5* is the putative transcriptional regulator of the cell wall integrity pathway (34), and upon its disruption in the presence of a *UPC2* GOF mutation, it is possible that the cell cannot effectively regulate the morphology of its cell wall and membrane. Also, the similarity of Cas5p- and Upc2p-dependent genes (discussed below) suggests cross talk between the two transcription factors. Studies to examine such a relationship may uncover other currently unknown roles for each TF.

The strain containing the *ERG11*$^{K143R}$ mutation had MICs in YPD medium of 4 μg/ml at 24 h and 8 μg/ml at 48 and 72 h. Although this background was not as highly resistant as others, its respective *CAS5* deletion mutant had 2-fold decreases in both the fluconazole MIC and MFC at 48 and 72 h. The *MRR1*$^{P683S}$ and *TAC1*$^{G980E}$ mutant fluconazole-resistant strains both had MICs in YPD medium of 8 μg/ml at 24 h, 16 μg/ml at 48 h, and >64 μg/ml at 72 h and MFCs of >64 μg/ml at all time points. These values decreased moderately in the respective *cas5*Δ/Δ mutants. A halo of reduced confluent growth was observed around the Etest strip for the constructed *MRR1*$^{P683S}$ and *TAC1*$^{G980E}$ mutants but not in the *ERG11*$^{K143R}$ background. Fitness defects have been shown to be associated with the introduction of resistance mutations and may explain the growth of the *ERG11*$^{K143R}$ mutant when examined by Etest (48). Despite the absence of reduced confluent growth, the *cas5*Δ/Δ mutants in each background showed increased susceptibility to fluconazole by Etest and a decreased ability to grow in the presence of fluconazole at 10 μg/ml. Although the loss of *CAS5* in strains containing a single mechanism of resistance resulted in slightly enhanced susceptibility to fluconazole, this modest effect is not practical for clinical applications in overcoming resistance. The differences in susceptibilities observed for the mutants constructed in the azole-resistant clinical isolates versus the isogenic strains may be due to the possibility that clinical isolates have compensatory mutations mitigating any decrease in fitness resulting from such resistance mutations (48).

We then sought to determine if *CAS5* might influence fluconazole susceptibility through altered expression of the efflux pump genes *CDR1*, *CDR2*, and *MDR1*. The expression of *CDR1* was unchanged in the absence of *CAS5*, in all mutant backgrounds. However, *CDR2* expression was upregulated in three of the five backgrounds, and *MDR1* expression was upregulated in two of the five backgrounds. Therefore, the moderate effect on susceptibility observed upon disruption of *CAS5* did not appear to be due to altered expression of the efflux pumps.

Another hypothesis for the enhanced killing activity of fluconazole against the *cas5*Δ/Δ mutants is changes in cell wall structure. Previous reports have shown that when the cell wall integrity pathway is disrupted, specifically by disrupting the protein kinase gene *MKC1*, the cell exhibits increased susceptibility to fluconazole and caspofungin (12, 13). The upstream kinase Pkc1p phosphorylates Mkc1p in response to fluconazole. Cas5p has been postulated to be the functional equivalent of the *Saccharomyces cerevisiae* cell wall integrity downstream transcriptional regulator ScRlm1p (34). As previous reports have suggested that cell wall integrity is critical for the fungistatic effect of sterol biosynthesis inhibitors (49), this implies that when *CAS5* is disrupted, the integrity of the cell wall is compromised, which results in enhanced fluconazole killing activity. Indeed, expression levels of cell wall integrity genes measured by microarray analysis were reduced in the *cas5*Δ/Δ mutant compared to its parent strain, SC5314, upon exposure to fluconazole. The *PGA13*, *PGA31*, *RCA1*, *VPS7*, *VPS28*, orf19.5644, and orf19.719 genes are all proposed to be involved in cell wall integrity. Interestingly, the gene ontology process categories of iron ion transport and iron ion homeostasis were also found to be dependent upon *CAS5*. This is consistent with the involvement of some cell wall proteins in iron acquisition (50).

Previous studies by our group also identified a similar set of iron ion transport and iron ion homeostasis genes to be dependent upon *UPC2* (20), suggesting cross talk between the two transcription factors. Indeed, upon disruption of *CAS5*, the baseline expression level of *ERG11* was reduced in isolate SC5314, as measured by real-time RT-PCR. Additionally, several genes involved in the lipid metabolic process were also identified to be Cas5 dependent, including *ERG26* of the ergosterol biosynthesis pathway, a process that is regulated by Upc2 (33, 45). *ERG26* encodes C-4 sterol decarboxylase and has been found to be an essential gene in *C. albicans* (51). Therefore, inhibition of this step may represent a potential drug target and warrants further investigation.

These data suggest that the enhanced killing activity of fluconazole observed in both susceptible and resistant strains lacking *CAS5* may be due to deregulation of iron ion transport and *ERG26* expression, in addition to the inability to upregulate genes involved in the cell wall integrity pathway. Delineation of which downstream targets influence azole killing activity would identify potential targets for cotherapeutic strategies rendering enhanced killing activity to the azoles and, as a result, may mitigate the development of resistance. Moreover, the known hypersusceptibility to caspofungin of *C. albicans* strains lacking *CAS5* suggests that inhibitors of this pathway may be valuable as cotherapeutic agents administered in tandem with the echinocandins as well (34).

## ACKNOWLEDGMENTS

This research was supported in part by a grant from the Children's Foundation Research Center at Le Bonheur Children's Hospital, Memphis, TN (E.M.V.), and National Institutes of Health grant R01AI058145 (P.D.R.).

We are grateful to Joachim Morschhäuser, Spencer Redding, and Ted White for mutant strains and clinical isolates. We thank Qing Zhang for her invaluable assistance in the laboratory.

We have no financial or commercial conflicts of interest to declare.

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