Gain-of-Function Mutations in \textit{UPC2} Are a Frequent Cause of \textit{ERG11} Upregulation in Azole-Resistant Clinical Isolates of \textit{Candida albicans}

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In \textit{Candida albicans}, Upc2 is a zinc-cluster transcription factor that targets genes, including those of the ergosterol biosynthesis pathway. To date, three documented \textit{UPC2} gain-of-function (GOF) mutations have been recovered from fluconazole-resistant clinical isolates that contribute to an increase in \textit{ERG11} expression and decreased fluconazole susceptibility. In a group of 63 isolates with reduced susceptibility to fluconazole, we found that 47 overexpressed \textit{ERG11} by at least 2-fold over the average expression levels in 3 unrelated fluconazole-susceptible strains. Of those 47 isolates, 29 contained a mutation in \textit{UPC2}, whereas the remaining 18 isolates did not. Among the isolates containing mutations in \textit{UPC2}, we recovered eight distinct mutations resulting in putative single amino acid substitutions: G648D, G648S, A643T, A643V, Y642F, G304R, A646V, and W478C. Seven of these resulted in increased \textit{ERG11} expression, increased cellular ergosterol, and decreased susceptibility to fluconazole compared to the results for the wild-type strain. Genome-wide transcriptional analysis was performed for the four strongest Upc2 amino acid substitutions (A643V, G648D, G648S, and Y642F). Genes commonly upregulated by all four mutations included those involved in ergosterol biosynthesis, in oxidoreductase activity, the major facilitator efflux pump encoded by the \textit{MDR1} gene, and the uncharacterized ATP binding cassette transporter \textit{CDR1}. These findings demonstrate that gain-of-function mutations in \textit{UPC2} are more prevalent among clinical isolates than previously thought and make a significant contribution to azole antifungal resistance, but the findings do not account for \textit{ERG11} overexpression in all such isolates of \textit{C. albicans}.

\textit{Candida albicans} is an opportunistic fungal pathogen that causes mucosal, cutaneous, and systemic infections, including oropharyngeal candidiasis (OPC), the most frequent infection in people with AIDS (9, 13). In the United States, Candida is the fourth-most-common organism isolated from nosocomial bloodstream infections and is associated with a mortality rate approaching 40% (24). Fluconazole and other azole antifungal agents have proven effective in the management of OPC; however, with increased use of these agents, treatment failures have occurred that have been associated with the emergence of azole-resistant strains of \textit{C. albicans} (25a). Theazole class of antifungals work by inhibiting the cytochrome P450 enzyme lanosterol demethylase, a critical enzyme in the synthesis of ergosterol which is encoded by the \textit{ERG11} gene (14). The efficacy of fluconazole is decreased in clinical isolates of \textit{C. albicans} by the interplay of several mechanisms of resistance (17, 21, 23, 32). Overexpression of the efflux transporter genes \textit{CDR1}, \textit{CDR2}, and \textit{MDR1} is a common mechanism of drug resistance in this organism (10, 17, 26). Point mutations in the \textit{ERG11} gene result in reduced binding affinity of azoles to their target without precluding enzymatic function (31). In addition to point mutations, overexpression of \textit{ERG11} has also been shown to decrease fluconazole susceptibility. \textit{ERG11} gene amplification by chromosome 5 duplication or the presence of a chr5L isochromosome is known to contribute toazole resistance (29). Alternately, the zinc-cluster transcription factor Upc2 has been shown to regulate the expression of \textit{ERG11} and other genes involved in ergosterol biosynthesis (19, 30). Previous studies show that activating mutations in Upc2 result in increased expression of \textit{ERG11} and decreased fluconazole susceptibility; however, only three substitutions (G648D, A643T, and A643V) in Upc2 have been identified in clinical isolates that contribute toazole resistance (7, 11, 12). In all cases, these substitutions have been identified in fluconazole-resistant clinical isolates of genetically matched isolate pairs.

In this study, we examined the prevalence of overexpression of \textit{ERG11}, as well as the overexpression of efflux pump genes \textit{CDR1}, \textit{CDR2}, and \textit{MDR1}, in a group of 63 unrelated fluconazole-resistant clinical isolates. We also investigated the prevalence of \textit{UPC2} mutations among isolates that overexpress \textit{ERG11}. We determined which \textit{UPC2} mutations, both previously described and novel, result in increased \textit{ERG11} expression, altered cellular ergosterol content, and increased resistance to azoles, as well as to terbinafine. Finally, we identified genes that are coordinately differentially expressed in four strains expressing unique \textit{UPC2} gain-of-function mutations.

**MATERIALS AND METHODS**

**Strains and growth conditions.** All \textit{C. albicans} strains (Table 1) were stored as frozen stock in 20% glycerol at $-80\degree$C and cultured on YPD (1% yeast extract, 2% peptone, and 1% dextrose) agar plates at 30°C. YPD liquid medium was used for routine growth of strains. For selection of strains containing the SAT1-flipper cassette (25), nourseothricin (200 μg/ml) was added to YPD agar plates. One Shot \textit{Escherichia coli} TOP10 chemically competent cells (Invitrogen, Carlsbad, CA) were used as the host for plasmid construction and propagation. These strains were grown in Luria-Bertani (LB) broth or on LB agar plates supplemented with 100

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\[
\text{Absorbances of subtracted from the sample cycle threshold (by using software provided with the 7000 sequence detection system. The association curve and detection of SYBR green fluorescence was performed activation at 95°C for 10 min, followed by 40 cycles of denaturation at 98°C.}
\]

\[1290\]

\[\text{H9262} \]

\[\text{/H9004} \]

\[\text{/H11002}/\text{H9004}/\text{H9004} \]

\[\text{SC5314} \]

\[\text{N/A} \]

\[\text{UPC2-1/UPC2-2} \]

\[\text{Azole susceptible} \]

\[\text{University of Iowa} \]

\[\text{Azole resistant} \]

\[\text{University of Iowa} \]

\[\text{H2O}. \]

\[\text{Quantity and purity were determined spectrophotometrically at 280.} \]

\[\text{RNA isolation. RNA was isolated using a small-scale version of the hot phenol method of RNA isolation described by Schmitt et al. (27). Briefly, overnight cultures were diluted to an optical density at 600 nm (OD}_{600} of 0.2 and then incubated at 30°C with shaking for an additional 3 or 6 h to mid-log phase. Cells were collected by centrifugation, resuspended in sodium acetate-EDTA buffer, and then transferred to a 2 mL microcentrifuge tube containing acid phenol (pH 4.3) with 1% SDS. Cells were incubated at 65°C for 10 min, and then lysates were subjected to centrifugation. The supernatant was then transferred into a new tube containing 900 μL of chloroform and mixed. The sample was then subjected to centrifugation again, and the aqueous layer was transferred to a new tube containing 1 volume of isopropanol and 0.1 volume of 2 M sodium acetate. The RNA pellet was subsequently washed with 500 μL of 70% ethanol and collected by centrifugation. The RNA pellet was resuspended in DNase/RNase-free H2O. Quantity and purity were determined spectrophotometrically at absorbances of A_{260} and A_{280}.} \]

\[\text{qRT-PCR. First-strand cDNAs were synthesized separately from 1 μg of total RNA in a 21-μL reaction mixture using the SuperScript first-strand synthesis system for reverse transcription (Invitrogen). Quantitative PCRs (qRT-PCRs) were performed in triplicate as technical replicates using the 7000 sequence detection system (Applied Biosystems). PCRs were performed, independently amplifying 18S rRNA or the ACT1 gene (normalizing genes) and the genes of interest (GOI) from the same cDNA, using SYBR green PCR master mix (Applied Biosystems).} \]

\[\text{TABLE 1 C. albicans strains used in this study} \]

| Strain | Strain background | Relevant characteristics or genotype | Source or reference |
|--------|------------------|--------------------------------------|---------------------|
| SC5314 | N/A              | UPC2-1/UPC2-2                        | ATCC                |

\[\text{Clinical isolates} \]

| 1–10  | N/A              | Azole susceptible                    | University of Iowa |
| 11–72 | N/A              | Azole resistant                      | University of Iowa |

\[\text{Constructed laboratory strains} \]

| UPC2M4A | SC5314 | UPC2-1/UPC2-2                        |                     |
| 11A8A2A | UPC2M4A | UPC2-2                               |                     |
| SC11A1A | SC5314 | UPC2-2                               |                     |
| 22A1A13A | UPC2M4A | UPC2-2                               |                     |
| SC22A3A | SC5314 | UPC2-2                               |                     |
| YFA3A2KI | UPC2M4A | UPC2-2                               |                     |
| SCYFA2A | SC5314 | UPC2-2                               |                     |
| AVA1A16A | UPC2M4A | UPC2-2                               |                     |
| SCAVA4  | SC5314 | UPC2-2                               |                     |
| 25B2D1  | UPC2M4A | UPC2-2                               |                     |
| SC25A1  | SC5314 | UPC2-2                               |                     |
| 28A7A10A | UPC2M4A | UPC2-2                               |                     |
| SC28A3A | SC5314 | UPC2-2                               |                     |
| SCUPC2R12A | SC5314 | UPC2-2                               |                     |
| SCUPC2R14A | SC5314 | UPC2-2                               |                     |
| SCUPC2R23A | SC5314 | UPC2-2                               |                     |
| SCUPC2R34A | SC5314 | UPC2-2                               |                     |

\[\text{μg/ml ampicillin (Sigma) or 50 μg/ml kanamycin (Fisher BioReagents, Fair Lawn, NJ) when required.} \]

\[\text{RNA isolation. RNA was isolated using a small-scale version of the hot phenol method of RNA isolation described by Schmitt et al. (27). Briefly, overnight cultures were diluted to an optical density at 600 nm (OD}_{600} of 0.2 and then incubated at 30°C with shaking for an additional 3 or 6 h to mid-log phase. Cells were collected by centrifugation, resuspended in sodium acetate-EDTA buffer, and then transferred to a 2 mL microcentrifuge tube containing acid phenol (pH 4.3) with 1% SDS. Cells were incubated at 65°C for 10 min, and then lysates were subjected to centrifugation. The supernatant was then transferred into a new tube containing 900 μL of chloroform and mixed. The sample was then subjected to centrifugation again, and the aqueous layer was transferred to a new tube containing 1 volume of isopropanol and 0.1 volume of 2 M sodium acetate. The RNA pellet was subsequently washed with 500 μL of 70% ethanol and collected by centrifugation. The RNA pellet was resuspended in DNase/RNase-free H2O. Quantity and purity were determined spectrophotometrically at absorbances of A_{260} and A_{280}.} \]

\[\text{Plasmid construction for allele sequencing. C. albicans UPC2 coding sequences were amplified by PCR (Pfu DNA polymerase; Stratagene) using a small-scale version of the hot phenol method of RNA isolation described by Schmitt et al. (27).} \]

\[\text{TABLE 2 Primers used in this study} \]

| Purpose, primer | Sequencea |
|-----------------|-----------|
| qRT-PCR         |           |
| ACT1-F          | 5'-ACGGTGAGAAGAGGTTGCTGTTATGTT-3' |
| ACT1-R          | 5'-CGATCGAAGGATATGGTGCTGTTATGTT-3' |
| 18S-F           | 5'-CACGAGCAGGATGTTCAACAAGA-3' |
| 18S-R           | 5'-CGATCGAAGGATATGGTGCTGTTATGTT-3' |
| ERG1I-F         | 5'-CCCTATATTTTTTTTCTTTTTTATATAT-3' |
| ERG1I-R         | 5'-ACGTCGTGCTCTTCTCAGTTATTTTTTATAT-3' |
| CDR1-F          | 5'-ATTCATTGAGTTGTCGCGAAGATG-3' |
| CDR1-R          | 5'-AGTTCTGCTGAAAATATTCGTAAGTTTATTT-3' |
| CDR2-F          | 5'-TAGTCCTGGATACGGACGAACT-3' |
| CDR2-R          | 5'-GACCAATGGTATTGGTTATCAGACAT-3' |
| BMR1-F          | 5'-ACAATAAACATTGTGGCCATCCAGAGA-3' |
| BMR1-R          | 5'-AACATGGTATTGGTTATCAGACAT-3' |
| UPC2 mutant construction |           |
| UPC2-A          | 5'-GGGCGGGAGATGTTGTGATTAGTTTAG-3' |
| UPC2-B          | 5'-CTCGGACTATATTCTATTGTGATTAGTTTAG-3' |
| UPC2-E          | 5'-CTCGGACTATATTCTATTGTGATTAGTTTAG-3' |
| UPC2-C          | 5'-GCGCGGAGATGTTGTGATTAGTTTAG-3' |
| UPC2-D          | 5'-GAGCTGCTTCTTCTAGTTGATTAGTTTAG-3' |
| UPC2 sequencing |           |
| UPC2seqA        | 5'-CTCGGACTATATTCTATTGTGATTAGTTTAG-3' |
| UPC2seqB        | 5'-CTCGGACTATATTCTATTGTGATTAGTTTAG-3' |
| UPC2seqC        | 5'-CTCGGACTATATTCTATTGTGATTAGTTTAG-3' |
| UPC2seqD        | 5'-CTCGGACTATATTCTATTGTGATTAGTTTAG-3' |
| UPC2seqZ        | 5'-CTCGGACTATATTCTATTGTGATTAGTTTAG-3' |
| UPC2seqF        | 5'-CTCGGACTATATTCTATTGTGATTAGTTTAG-3' |
| UPC2seqG        | 5'-CTCGGACTATATTCTATTGTGATTAGTTTAG-3' |
| UPC2seqH        | 5'-CTCGGACTATATTCTATTGTGATTAGTTTAG-3' |

a Underlined sequence reflects the introduction of a restriction site sequence.
from *C. albicans* genomic DNA using the primers UPC2-A and UPC2-E (Table 2). Products were cloned into pCR-BLUNTII-TOPO using a Zero Blunt TOPO PCR cloning kit (Invitrogen) and transferred into *Escherichia coli* TOP10 cells with selection on LB agar plates containing 50 µg/ml kanamycin. Plasmid DNA was purified (QIAprep; Qiagen, Germantown, MD) and sequenced on an ABI model 3130XL genetic analyzer using the UPC2 sequencing primers (Table 2), resulting in a full-length sequence from both strands of the *C. albicans* UPC2 gene. The sequencing was performed using six sets of clones derived from three independent PCRs for each strain/isolate sequenced.

Sequenced plasmids containing the UPC2 open reading frame (ORF) whose predicted translation indicated an amino acid substitution were digested with restriction enzymes Apal and Xhol, which excised the full-length ORF from the plasmid, and the UPC2 alleles were cloned upstream of the SAT1-flipper cassette into the Apal and Xhol sites of plasmid pSFS2 (25). The UPC2 downstream segments were amplified with *Ex Taq* (Takara) using primers UPC2C and UPC2D and cloned downstream of the SAT1-flipper cassette in pSFS2 using the SacI and SacII sites. This process generated plasmids pUPC2-G648S, pUPC2-G309R, pUPC2-Y642F, and pUPC2-Y646V.

**Construction of UPC2 allele strains.** *C. albicans* strains UPC2M4A (7) and SC5314 were transformed by electroporation with gel-purified inserts from plasmids pUPC2-G648S, pUPC2-M597I, pUPC2-G309R, pUPC2-A643V, pUPC2-Y642F, pUPC2-W467C, and pUPC2-A646V derived from the plasmid pSFS2. pSFS2 contains the SAT1-flipper disruption cassette developed by Reuss et al. (25), consisting of the SAT1 selectable marker which confers resistance to nourseothricin and the *FLP* flipper recombinase gene, both flanked by FRT sites (flipper recombinase target sequences). Nourseothricin-resistant transformants were selected as previously described (25). Upon induction of the FLP gene, the cassette is excised such that only the UPC2 allele with a downstream FRT is left in the UPC2 locus. Integration of constructs was confirmed by Southern hybridization.

**Fluconazole susceptibility testing.** MICs were obtained by using a modified CLSI protocol outlined in CLSI document M27-A3 using RPMI or YPD medium (1). Overnight cultures grown at 30°C were streaked onto Sabouraud’s agar. Plated cultures were grown for 24 h at 30°C. Individual colonies were suspended in sterile water until an OD600 of 0.1 was reached. The working colony concentration was made by multiplying 1.5 and 2 dilution and 1:20 dilution sequentially in medium. Aliquots of 100 µl from the working stock were used to inoculate a series of fluconazole-YPD medium dilutions, the highest being 64 µg/ml. Similar procedures were used for terbinafine and amphotericin B dilutions; however, the highest concentration used for these agents was 8 µg/ml. Cultures were incubated at 35°C for 48 h, and MICs were recorded. Azole MICs were unrelated, and they were collected from both oral and non-oral sources, including blood, tissue, and abdominal fluid (see Table S1 in the supplemental material). Fluconazole susceptibilities were confirmed in our laboratory. We defined all isolates with a MIC of ≥16 µg/ml as having resistance to fluconazole for the purpose of this study. From our collection of 72 isolates, 9 were classified as fluconazole susceptible, while the remaining isolates were resistant. We then measured the levels of expression of *ERG11*, *CDR1*, *CDR2*, and *MDR1* for all isolates in order to determine the relative prevalence of overexpression of genes indicative of specific mechanisms of azole resistance (Fig. 1). We defined overexpression as an increase in expression of at least 2-fold. We observed that 77% (n = 49) of resistant isolates overexpressed both *CDR1* and *CDR2*. *MDR1* overexpression was increased by at least 2-fold in 76% (n = 48) of resistant isolates, but only 21% (n = 13) expressed *MDR1* at levels consistent with the levels observed in isolates with *MDR1*-mediated fluconazole resistance, in which *MDR1* expression is substantially increased. For example, in a study by Morschhäuser et al., the clinical isolates examined exhibited 400- to 1,200-fold increases in *MDR1* expression (22). Surprisingly, *ERG11* was found to be overexpressed in 75% (n = 47) of fluconazole-resistant isolates in this collection.
Most but not all isolates that overexpress \textit{ERG11} carry a GOF mutation in \textit{UPC2}. Twenty-nine of the 47 isolates that overexpressed \textit{ERG11} possessed one or both \textit{UPC2} alleles with a mutation that resulted in a predicted amino acid substitution. Although many silent mutations were observed in the \textit{UPC2} alleles tested, eight distinct single \textit{UPC2} mutations were also recovered, three of which had been described previously: G648D, A643T, and A643V (Table 3). Eighteen clinical isolates were found to overexpress \textit{ERG11} yet contained no amino acid substitution in Upc2. These data suggest an alternate mechanism of activating \textit{ERG11} expression in these isolates.

**FIG 1** Expression levels of \textit{CDR1}, \textit{CDR2}, \textit{MDR1}, and \textit{ERG11} in 63 fluconazole-resistant clinical isolates were measured by qRT-PCR. All gene expression levels were measured in triplicate, and fold expression of genes in resistant isolates was compared to the average of the expression levels in three susceptible isolates. Results for 63 isolates with reduced susceptibility to fluconazole are represented, but only even-numbered isolates’ results are labeled. Error bars show standard errors.

GOF mutations in \textit{UPC2} result in increased expression of \textit{ERG11}. In order to assess the contribution of each individual mutant \textit{UPC2} allele to fluconazole resistance, we expressed each mutant allele alone, with the other \textit{UPC2} allele disrupted (data not shown), as a heterozygote with a wild-type \textit{UPC2} allele, and as a homozygote. \textit{ERG11} mRNA abundance was measured by qRT-PCR (Fig. 2). The homozygous null upc2Δ strain showed a significant decrease in \textit{ERG11} expression compared to that of its parental wild-type strain. Seven of the eight mutations tested resulted in increased \textit{ERG11} expression, with the strongest expression observed among homozygous strains.
Interestingly, different mutations elicited different levels of \( \text{ERG11} \) expression, with the G648D substitution resulting in the highest level of expression.

**GOF mutations in \( \text{UPC2} \) result in elevated cellular ergosterol content.** In order to confirm that activation of Upc2 results in a global increase of ergosterol biosynthesis, we compared the cellular ergosterol content in the wild type to the amounts in derivatives that were heterozygous or homozygous for various UPC2 mutations. We found that strains carrying \( \text{UPC2} \) GOF alleles generally contained larger amounts of ergosterol than the wild-type strain (Fig. 3). For many isolates, increased ergosterol levels correlated with the increased \( \text{ERG11} \) transcript levels resulting from each individual \( \text{UPC2} \) mutation; however, this was not the case for the A643V allele, which resulted in strong \( \text{ERG11} \) expression but a nonsignificant increase in cellular ergosterol content. \( \text{UPC2} \) alleles containing the G648D, W478C, and Y642F amino acid substitutions showed statistically significant increases in ergosterol content relative to the amount in the wild-type strain, while strains carrying other \( \text{UPC2} \) GOF alleles trended toward larger amounts of cellular ergosterol.

**GOF mutations in \( \text{UPC2} \) influence susceptibility to antifungals.** To examine the impact of distinct \( \text{UPC2} \) mutations on antifungal resistance, we determined the susceptibilities of strains constructed to carry mutant \( \text{UPC2} \) alleles to a panel of antifungals (Table 4). Included in this panel were agents in the azole class (fluconazole, itraconazole, and voriconazole). Also included were terbinafine, which inhibits another ergosterol biosynthesis enzyme (squalene epoxidase), and amphotericin B, which targets ergosterol in the fungal cell membrane. All strains constructed

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**TABLE 3 Occurrence of Upc2 amino acid substitutions in predicted translated sequence in fluconazole-resistant clinical \( \text{ERG11} \)-overexpressing isolates**

| Upc2 substitution | Isolate | Genotype   | Fluconazole MIC (µg/ml) | Expression (fold) of efflux transporter gene (±SE)* |
|-------------------|---------|------------|-------------------------|-----------------------------------------------|
|                   |         |            |                         | \( \text{ERG11} \) | \( \text{CDR1} \) | \( \text{CDR2} \) | \( \text{MDR1} \) |
| G648D             | 21      | Heterozygous | 32                      | 6.2 (±0.2) | 14.7 (±5.5) | 883.3 (±301.9) | 10.4 (±3.1) |
|                   | 38      | Heterozygous | 64                      | 3.8 (±0.3) | 3.9 (±0.7)  | 111.0 (±57.1) | 1.4 (±0.2)  |
|                   | 47      | Heterozygous | 128                     | 7.9 (±1.2) | 11.8 (±5.1) | 327.0 (±166.3)| 8.2 (±3.1)  |
|                   | 52      | Heterozygous | 256                     | 3.7 (±1.4) | 1.2 (±0.3)  | 17.1 (±3.2)  | 0.3 (±0.1)  |
|                   | 59      | Heterozygous | >256                    | 7.2 (±1.0) | 6.5 (±0.3)  | 48.5 (±20.3) | 8.0 (±0.2)  |
|                   | 71      | Heterozygous | >256                    | 9.4 (±1.1) | 5.6 (±1.9)  | 149.8 (±56.3)| 4.6 (±1.8)  |
| G648S             | 25      | Heterozygous | 64                      | 3.6 (±0.6) | 1.1 (±0.3)  | 2.8 (±1.1)  | 160.5 (±78.9)|
|                   | 35      | Heterozygous | >256                    | 4.4 (±1.3) | 1.2 (±0.4)  | 0.7 (±0.3)  | 79.9 (±22.8)|
|                   | 55      | Homozygous  | >256                    | 2.6 (±0.6) | 1.7 (±1.3)  | 26.2 (±17.4) | 0.9 (±3.2)  |
|                   | 56      | Homozygous  | >256                    | 2.1 (±1.0) | 8.0 (±3.1)  | 167.8 (±94.0)| 2.5 (±0.9)  |
|                   | 57      | Homozygous  | >256                    | 19.4 (±12.4)| 10.2 (±3.5) | 263.3 (±106.0)| 12.9 (±5.2)|
|                   | 58      | Homozygous  | >256                    | 6.6 (±1.5) | 9.7 (±4.2)  | 226.6 (±131.7)| 3.8 (±1.2)|
|                   | 65      | Heterozygous | >256                    | 4.2 (±0.3) | 9.8 (±1.5)  | 294.9 (±128.4)| 2.4 (±0.1)|
|                   | 67      | Heterozygous | >256                    | 4.7 (±0.7) | 7.4 (±2.8)  | 105.1 (±43.3)| 1.2 (±0.5)|
|                   | 69      | Heterozygous | >256                    | 5.6 (±0.9) | 7.2 (±0.5)  | 136.8 (±69.3)| 1.4 (±0.5)|
| A643T             | 13      | Homozygous  | 16                      | 2.4 (±1.1) | 6.4 (±2.9)  | 185.9 (±80.9)| 2.4 (±1.6)|
| A643V             | 30      | Heterozygous | 64                      | 4.6 (±1.6) | 1.4 (±0.3)  | 18.0 (±215.7)| 18.9 (±216.7)|
|                   | 68      | Heterozygous | >256                    | 4.9 (±0.4) | 6.3 (±2.2)  | 64.5 (±31.8) | 216.3 (±98.9)|
| A646V             | 45      | Heterozygous | 128                     | 3.5 (±0.3) | 4.8 (±0.3)  | 147.8 (±71.7)| 15.5 (±7.7)|
|                   | 67      | Heterozygous | >256                    | 4.7 (±0.7) | 7.4 (±2.8)  | 105.1 (±43.3)| 1.2 (±0.5)|
| Y642F             | 33      | Homozygous  | 36                      | 12.6 (±1.8)| 6.8 (±2.0)  | 201.5 (±129.0)| 1.5 (±0.5)|
|                   | 34      | Homozygous  | 37                      | 7.5 (±0.6) | 5.4 (±2.5)  | 126.8 (±62.6)| 1.2 (±0.5)|
| W478C             | 15      | Heterozygous | 16                      | 8.1 (±2.7)| 15.9 (±3.8)| 1415.1 (±400.1)| 4.0 (±3.0)|
|                   | 27      | Heterozygous | 64                      | 6.2 (±2.8)| 14.2 (±4.7)| 629.7 (±255.3)| 1.1 (±0.1)|
|                   | 28      | Heterozygous | 64                      | 18.1 (±7.3)| 18.1 (±6.3)| 1439.7 (±570.8)| 23.2 (±20.6)|
| G304R             | 18      | Heterozygous | 32                      | 5.6 (±1.2)| 10.4 (±2.4)| 575.0 (±117.2)| 1.0 (±0.3)|
|                   | 19      | Heterozygous | 32                      | 5.2 (±1.4)| 10.0 (±4.8)| 401.3 (±204.5)| 4.6 (±3.0)|

* qRT-PCR was performed in triplicate.

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**FIG 2** \( \text{ERG11} \) transcription levels in strains carrying mutant \( \text{UPC2} \) alleles are represented as averages from three independent qRT-PCR assays. Error bars show standard errors. \( \text{ERG11} \) expression was quantified for each mutant strain listed and is compared to that of wild-type strain SC5314.
FIG 3 Ergosterol quantification in *C. albicans* laboratory strains expressing mutant UPC2 alleles. Increased ergosterol content was shown for strains expressing 2 UPC2 mutant alleles compared to the expression levels in heterozygote and wild-type strains. The heptane extraction layer from 16-h cultures was scanned spectrophotometrically between 240 and 300 nm. The presence of ergosterol in an extracted sample resulted in a 4-peak curve. A decrease in absorbance peaks correlates to a decrease in ergosterol content. Each panel represents the results for a different Upc2 GOF mutation, as follows: G648S (A), A643V (B), Y642F (C), G648D (D), A646V (E), A643T (F), W478C (G), and G304R (H). UPC2 mutant alleles were expressed as homozygotes with the same GOF mutation (●) or as heterozygotes with the wild-type allele (▲). Their absorbencies were compared to those of the wild-type strain SC5314 (○). The results for the homozygous revertants shown in panels C, D, and G showed statistically significant greater ergosterol contents than were found in the wild type (P < 0.05).
were compared to the background strain, strain SC5314, and the upc2Δ/Δ mutant strain UPC2M4A. Strain UPC2M4A was highly susceptible to terbinafine, as well as all the azoles tested. Interestingly, this strain exhibited a 4-fold-higher MIC of amphotericin B than the eight mutations in the parental wild-type strain. However, all strains tested were generally demonstrated a reduction in MIC at 48 h compared to that of the parental wild-type strain, exhibiting a 4-fold-higher MIC of amphotericin B than strains homozygous for an activated UPC2 mutant allele. A similar trend was exhibited for the other azole agents and also for terbinafine susceptibilities. For amphotericin B, strains homozygous for an activated UPC2 mutant allele did not show a decrease in susceptibility beyond that of the wild-type, indicating that this mutation does not result in gain of function. Correlating with susceptibility beyond that of the wild-type, indicating that this mutation does not result in gain of function. 

**TABLE 4 MICs of a panel of antifungals in YPD against strains expressing UPC2 mutant alleles**

| Strain   | Relevant genotype | FCZ (µg/ml) | ICZ | VCZ | TBF | AMB |
|----------|-------------------|-------------|-----|-----|-----|-----|
| SC5314   | UPC2-1/UPC2-2     | 0.5 4       | 0.016 0.023 | 0.008 0.032 | 0.5 2 | 0.0313 0.125 |
| UPC2M4A  | upc2::FRT1/UPC2-2 | 0.023 0.032 | 0.002 0.004 | 0.002 0.002 | <0.016 <0.016 | 0.0313 0.5 |
| 11A8A2A  | UPC2G648S::FRT1/UPC2-2 | 4 >256 0.5 | 0.75 0.25 | 0.25 >32 | 2 4 | 0.016 0.125 |
| SC11A1A  | UPC2G648S::FRT1/UPC2-2 | 1 >256 0.032 | 0.19 0.047 | 0.47 >32 | 2 4 | 0.0625 0.25 |
| 28A7A10A | UPC2G648S::FRT1/UPC2-2 | 2 >256 0.125 >32 | 0.25 >32 | 1 4 | 0.016 0.125 |
| SC28A3A  | UPC2G648S::FRT1/UPC2-2 | 1 2 | 0.023 0.047 | 0.047 >32 | 1 2 | 0.0313 0.25 |
| YFA3A2K1 | UPC2G648S::FRT1/UPC2-2 | 2 >256 0.047 0.5 | 0.064 >32 | 1 4 | 0.0625 0.5 |
| SCYF2A2  | UPC2G648S::FRT1/UPC2-2 | 0.75 1 | 0.023 0.032 | 0.023 0.19 | 1 2 | 0.0313 0.25 |
| AVA1A16A | UPC2G648S::FRT1/UPC2-2 | 1.5 >256 0.064 0.25 | 0.064 >32 | 1 4 | 0.0313 0.25 |
| SCAVA4   | UPC2G648S::FRT1/SAT1-FLP | 0.5 1.5 | 0.016 0.032 | 0.016 0.064 | 1 2 | 0.0313 0.5 |
| 22A1A13A | UPC2G648S::FRT1/UPC2-2 | 0.19 0.75 | 0.012 0.023 | 0.008 0.032 | 1 2 | 0.0313 0.5 |
| SC22A3A  | UPC2G648S::FRT1/UPC2-2 | 0.75 >256 0.012 0.094 | 0.023 >32 | 1 4 | 0.0313 0.25 |
| 25B2D1   | UPC2G648S::FRT1/UPC2-2 | 1 2 | 0.047 0.047 | 0.016 0.5 | 0.25 1 | 0.0313 0.25 |
| SC25A1   | UPC2G648S::FRT1/UPC2-2 | 0.5 1 | 0.016 0.016 | 0.008 0.032 | 0.5 1 | 0.016 0.125 |
| SCUPC2R1A | UPC2G648S::FRT1/UPC2-2 | 8 >256 >32 >32 | 0.064 >32 | 2 4 | 0.016 0.125 |
| SCUPC2R12A | UPC2G648S::FRT1/UPC2-2 | 1.5 >256 >32 >32 | 0.047 >32 | 1 2 | 0.0313 0.25 |
| SCUPC2R34A | UPC2G648S::FRT1/UPC2-2 | 1.5 >256 2 >32 | 0.064 >32 | 2 2 | 0.016 0.125 |
| SCUPC2R32A | UPC2G648S::FRT1/UPC2-2 | 2 >256 0.016 0.032 | 0.012 >32 | 1 2 | 0.016 0.125 |

\* Susceptibility was tested at 24 and at 48 h. Azole MICs were determined by Etest method, while terbinafine and amphotericin B MICs were determined by broth microdilution methods. FCZ, fluconazole; ICZ, itraconazole; VCZ, voriconazole; TBF, terbinafine; AMB, amphotericin B.

**DISCUSSION**

In *Candida albicans*, it is well established that activating mutations in transcription factors regulating genes encoding efflux pumps mediate resistance to azole antifungals. It has been shown that specific mutations in the transcription factor gene TAC1 mediate the expression of the genes encoding ABC transporters CDR1 and CDR2, resulting in increased azole resistance in *C. albicans* (2, 3). Likewise, Mrr1 has been identified as the regulator of the major facilitator superfamily (MFS) transporter Mdr1 in azole-resistant isolates, and specific mutations in this transcriptional regulator result in its constitutive activation, leading to the overexpression of Mdr1 (6, 22). Fluconazole-resistant isolates that overexpress MDR1 have become homozogous for the mutated MRR1 allele and work in a semidominant fashion if expressed with a wild-type allele (6). Overexpression of these transporters is known to occur among azole-resistant clinical isolates and contributes significantly to this process.

Considerably less is known about the prevalence of constitutive ERG11 overexpression and its clinical impact on azole resistance. ERG11 encodes the azole target, lanosterol demethylase, a key enzyme in the ergosterol biosynthesis pathway. ERG11 is transcriptionally regulated by the zinc cluster transcription factor Upc2. In *C. albicans*, Upc2 is orthologous to two transcription factors in *Saccharomyces cerevisiae*, Upc2 and Ecm22, which regulate ergosterol biosynthesis and uptake of exogenous sterols (4,

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| Function                      | CGD name | orf19 designation | Change in expression (fold) | A643V |
|------------------------------|----------|-------------------|-----------------------------|-------|
| **Lipid metabolic process**  |          |                   | 12121212                    |       |
| ERG1                         | orf19.406| 1.8               | 1.8                         | 2.0   |
| ERG2                         | orf19.6026| 1.7               | 1.5                         | 1.9   |
| ERG24                        | orf19.1598| 1.7               | 1.7                         | 1.8   |
| ERG27                        | orf19.3240| 1.8               | 2.1                         | 2.2   |
| ERG7                         | orf19.1570| 1.5               | 1.6                         | 1.8   |
| NCP1                         | orf19.2672| 1.6               | 1.7                         | 1.7   |
| orf19.3483                   | 2.0      | 2.4               | 5.3                         | 2.9   |
| orf19.1881                   | 1.5      | 1.6               | 2.4                         | 1.5   |
| orf19.6025                   | 2.2      | 2.1               | 3.9                         | 5.2   |
| **Transcription factor activity** |         |                   | 1.8                         | 1.5   |
| MDR1                         | orf19.5604| 5.9               | 5.5                         | 6.6   |
| orf19.5535                   | 1.5      | 1.6               | 2.2                         | 1.5   |
| FRP1                         | orf19.5634| 5.4               | 5.7                         | 8.1   |
| FTH1                         | orf19.4802| 2.6               | 2.6                         | 5.2   |
| GYP1                         | orf19.3811| 1.6               | 1.6                         | 3.5   |
| orf19.2350                   | 1.7      | 1.7               | 3.4                         | 4.0   |
| orf19.4384                   | 1.8      | 2.1               | 2.6                         | 2.0   |
| **Transport**                |          |                   | 1.8                         | 1.5   |
| COQ4                         | orf19.3008| 1.5               | 1.5                         | 2.3   |
| HEM14                        | orf19.4747| 2.1               | 1.6                         | 3.0   |
| **Pathogenesis**             |          |                   | 1.8                         | 1.6   |
| SLD1                         | orf19.260| 1.8               | 1.5                         | 2.2   |
| PTO100                       | orf19.4424| 1.5               | 3.4                         | 4.8   |
| SET3                         | orf19.7221| 2.8               | 2.7                         | 6.3   |
| **Metabolic process**        |          |                   | 1.6                         | 2.5   |
| TEF4                         | orf19.2652| 1.6               | 16.6                        | 55.8  |
| orf19.3617                   | 1.7      | 1.9               | 1.7                         | 1.7   |
| orf19.329                    | 2.1      | 2.3               | 2.4                         | 2.6   |
| orf19.4031                   | 1.8      | 1.8               | 2.5                         | 2.5   |
| orf19.6025                   | 2.2      | 2.1               | 3.9                         | 5.2   |
| orf19.1865                   | 2.4      | 2.2               | 3.7                         | 2.9   |
| orf19.496                    | 2.4      | 2.3               | 4.4                         | 3.8   |
| ARO9                         | orf19.1237| 1.5               | 3.4                         | 5.8   |
| IDP2                         | orf19.3733| 2.2               | 1.6                         | 3.3   |
| **Response to stress**       |          |                   | 2.2                         | 2.5   |
| DDR48                        | orf19.4082| 2.2               | 4.3                         | 5.8   |
| FMA1                         | orf19.6837| 1.8               | 3.2                         | 2.7   |
| YMX6                         | orf19.5713| 1.6               | 2.6                         | 2.9   |
| orf19.288                    | 1.8      | 1.5               | 3.0                         | 2.5   |
| **Biological activity unknown** |       |                   | 16.4                        | 16.6  |
| TEF4                         | orf19.2652| 16.4              | 16.6                        | 55.8  |
| orf19.3627                   | 4.0      | 1.9               | 7.0                         | 4.0   |
| orf19.3627                   | 4.0      | 1.9               | 7.0                         | 4.0   |
| orf19.5635                   | 2.3      | 3.7               | 5.7                         | 2.4   |
| orf19.3094                   | 1.9      | 1.9               | 4.1                         | 3.1   |
| orf19.2451                   | 1.7      | 1.5               | 2.1                         | 2.2   |
| orf19.344                    | 5.1      | 6.2               | 20.2                        | 16.2  |
| orf19.5777                   | 1.7      | 2.4               | 4.8                         | 3.8   |
| orf19.5799                   | 1.7      | 2.0               | 3.4                         | 2.4   |
| orf19.7456                   | 1.7      | 2.6               | 4.4                         | 4.1   |
| orf19.7043                   | 2.5      | 1.9               | 5.2                         | 3.6   |
| orf19.4013                   | 2.6      | 2.2               | 4.2                         | 3.5   |
| orf19.4014                   | 1.5      | 2.1               | 2.3                         | 2.4   |
| orf19.6840                   | 2.0      | 2.1               | 4.1                         | 4.4   |
| orf19.286                    | 2.1      | 2.5               | 2.3                         | 2.2   |
| orf19.7504                   | 2.1      | 1.8               | 3.0                         | 3.3   |
| orf19.3737                   | 1.6      | 1.9               | 2.9                         | 3.4   |
| orf19.1964                   | 2.0      | 1.8               | 2.5                         | 2.2   |
| orf19.1800                   | 2.3      | 2.2               | 4.6                         | 3.9   |
| orf19.7263                   | 1.8      | 1.9               | 2.9                         | 2.8   |
| orf19.2496                   | 1.7      | 1.8               | 2.6                         | 2.5   |
| orf19.3261                   | 1.8      | 2.2               | 2.7                         | 3.4   |

* Descriptions of genes are from the Candida Genome Database (http://www.candidagenome.org).

* Fold change is defined as the average ratio of gene expression levels in the isolates compared in two independent microarray experiments.
The expression of these genes is affected by sterol depletion, but the exact mechanism of activation is currently unknown. Previous work in *S. cerevisiae* shows that ScUpc2 and ScEcm22 are localized to intracellular membranes outside the nucleus, and under sterol-depleting conditions, perinuclear localization is increased (20). Gain-of-function mutations in the activation domain located near the C terminus have been recovered from both ScUpc2 and ScEcm22; however, mediation of *ERG11* expression during hypoxic conditions or when chemically treated withazole antifungals is mediated by ScUpc2 (5).

In *C. albicans*, only three mutations that increase the expression of *ERG11* and result in decreased susceptibility to fluconazole have been described in Upc2. In the present study, we examined a large collection of unrelated azole-resistant *C. albicans* clinical isolates for possible *UPC2* GOF mutations. As fluconazole resistance in *C. albicans* has most often been associated with OPC, the opportunity to examine resistant isolates cultured from both oral and nonoral sources was somewhat unique. Among the isolates studied here were 16 documented nonoral isolates, 12 of which were observed to exhibit reduced susceptibilities to fluconazole.

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We further delineated the mechanisms of resistance in these isolates by determining the relative levels of expression of *CDR1*, *CDR2*, *MDR1*, and *ERG11*. Not surprisingly, *CDR1* and *CDR2* overexpression was generally coordinately regulated and quite prevalent among these isolates. Of those isolates that did overexpress *MDR1*, even fewer isolates expressed *MDR1* to the levels previously observed in azole-resistant isolates (22). *ERG11* was found to be upregulated in almost three-fourths of the fluconazole-resistant isolates examined. This suggests that *ERG11* overexpression is a common contributor to fluconazole resistance in *C. albicans*.

Among the independent azole-resistant, *ERG11*-overexpressing isolates studied here, we repeatedly recovered eight distinct single-nucleotide substitutions in *UPC2*, of which the G648S substitution occurred in nine isolates and the G648D substitution occurred in six isolates. Five of these substitutions in *UPC2* have not been described previously. Of the five novel mutations, four mutations resulted in increased *ERG11* expression and increased resistance to fluconazole but to various degrees. We observed that the homozygous strain carrying the G304R *UPC2* allele was more susceptible to azoles than the strain carrying the G304R substitution heterozygously; however, neither strain resulted in any decrease in antifungal susceptibility or increase in *ERG11* expression. As has been previously observed in *UPC2* GOF mutations, amino acid substitutions resulting from activating mutations were localized near the C terminus, where the activation domain of zinc-cluster transcription factors is found (18). Mutations in this region of the protein are theorized to work by one of two mechanisms: (i) mutations in this domain could relieve the transcription factor from a repressor that would otherwise keep *Upc2* inactive in the absence of an activating signal or (ii) a mutation in this area could interfere with the transmembrane region of the protein, causing localization to the nucleus and constitutive activation of its target promoters.

In total, seven of the eight Upc2 mutations observed resulted in increased expression of *ERG11*, but each mutation increased transcription to a different degree. For many strains, increased expression of *ERG11* was directly correlated with the amount of ergosterol within the fungal cell, and this trend was also observed in the decreased azole susceptibility seen in strains that overexpressed *ERG11*. Unlike *TAC1* GOF mutations, *UPC2* GOF mutations were found to occur with a wild-type allele in many isolates. We observed that strains constructed to express two mutant alleles had increased levels of *ERG11* expression and decreased levels ofazole susceptibility compared to the levels in strains containing both an activated *Upc2* and a wild-type allele. As seen with *TAC1* or *MDR1* mutations, a loss-of-heterozygosity event that results in two GOF alleles, either by a mitotic recombination event between homologous chromosomes or by a loss of a chromosome combined with a duplication of the homologous chromosome, would provide an advantage when challenged with antifungals (2, 22). Interestingly, we saw an opposite trend in the amphotericin B susceptibility of strains expressing activated *UPC2* alleles, in which *UPC2* GOF homoygous strains with higher *ERG11* expression had increased susceptibility to this agent compared to that of the heterozygous strains or the homoygous null *upc2Δ* strain. This is probably due to higher levels of cellular ergosterol, which is the target of amphotericin B.

A new observation of note is the discrepancy between the levels of *ERG11* expression of the clinical isolate containing a *UPC2* mutation and the laboratory strain constructed to carry the same mutant *UPC2* allele. This discrepancy may be due to additional levels of *ERG11* regulation that are developed in a clinical isolate, such as alternate transcriptional regulations or alternate single-nucleotide polymorphisms that influence ergosterol biosynthesis. Furthermore, not all *ERG11* overexpression in clinical isolates was a result of a GOF mutation in *UPC2*; therefore, it is likely that other regulators of ergosterol biosynthesis are contributing factors. Alternatively, instances of aneuploidy, specifically,

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**TABLE 6 Genes downregulated by at least 1.5-fold in strains carrying UPC2 GOF alleles**

| Function | CGD name | orf19 designation | Change in expression (fold) in indicated experiment |
|----------|----------|------------------|--------------------------------------------------|
| Transport | HGT10    | orf19.5753       | G648S G648D Y642F A643V |
| Pathogenesis | SAP7    | orf19.756        | 0.6 0.5 0.2 0.2 0.5 0.6 0.2 0.3 |
| Biological activity | unknown | orf19.716        | 0.4 0.4 0.3 0.2 0.4 0.4 0.3 0.4 |
|          |          | orf19.3621       | 0.3 0.5 0.3 0.3 0.6 0.4 0.5 0.5 |
|          |          | orf19.4873       | 0.4 0.5 0.6 0.5 0.6 0.6 0.6 0.6 |

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

b Fold change is defined as the average ratio of gene expression levels in the isolates compared in two independent microarray experiments.
chromosome 5 duplication or chr5L isochromosome formation, are known mechanisms of ERG11 overexpression in C. albicans (29). However, the ERG11 expression levels we observed in many clinical isolates in this study exceed what would be expected from an extra copy of ERG11 due to the presence of a chr5L isochromosome. It is possible that some isolates that do not carry mutations in UPC2 actually overexpress UPC2 instead, due to a mutation in the UPC2 promoter region, gene duplication, or a trans-acting mutation. Whether this has occurred is being explored in ongoing investigations.

As azole resistance is mechanistically multifactorial in clinical isolates, we expected the fluconazole susceptibility of strains constructed to express specific UPC2 mutations to reflect the contribution of increased expression of genes of the ergosterol biosynthesis pathway, including ERG11, as a result of an activated UPC2 allele. In these clinical isolates, we have observed a high prevalence of mutations in the ERG11 gene itself (data not shown). It is likely that in clinical isolates, activated forms of Upc2 occur in conjunction with mutant ERG11 alleles and that its subsequent overexpression results in a combinatorial effect on azole susceptibility. The combinatorial effects of activated UPC2 alleles and mutant ERG11 alleles on fluconazole susceptibility have not been explored.

Although microarray analysis of the G648D UPC2 GOF mutation has been performed previously, we expanded this analysis with four of the seven strongest UPC2 GOF mutations (G648D, G648S, Y642F, and A643V) for a more comprehensive examination (7). All four mutations caused the coordinate upregulation of 61 genes. As expected, many of the ergosterol biosynthesis genes (ERG1, ERG2, ERG7, ERG24, ERG26, and ERG27) were upregulated by at least 1.5-fold over their expression in SC5314 in two separate experiments. ERG11 was upregulated in three of the four laboratory strains carrying mutations G648D, Y642F, and A643V, but elevated transcription of ERG11 could only be detected in one of the two experiments with the strain carrying the G648S allele (see Table S2 in the supplemental material). Interestingly, UPC2 was not found to be upregulated in any of the four strains analyzed by microarray analysis, although Upc2 is thought to autoregulate and has been shown to bind its own promoter in experiments using chromatin immunoprecipitation with microarray technology (ChIP-chip) (33).

Aside from genes involved in the ergosterol biosynthesis pathway, other core sets of target genes upregulated by Upc2 included those in the gene ontology (GO) functional group of oxidoreductase activity. Additionally, iron ion binding was a commonly found GO category identified by the GO term finder. Not surprisingly, MDR1 was overexpressed in all four strains with independent Upc2 GOF mutations; however, gene expression levels were not increased to those observed in fluconazole-resistant iso-

In conclusion, we found overexpression of ERG11 to be prevalent among a collection of clinical isolates with reduced susceptibility to fluconazole and have identified and characterized novel UPC2 gain-of-function mutations that contribute to azole resistance in clinical isolates of C. albicans. Overexpression of ERG11 could be explained by a gain-of-function mutation in UPC2 in many but not all cases. Gain-of-function mutations led to increased resistance to azole antifungals and terbinafine, to increased cellular ergosterol levels, and to increased expression of genes involved in ergosterol biosynthesis and oxidoreductase activity, as well as the transporter genes MDR1 and CDR1. The mechanism by which ERG11 is upregulated in the absence of UPC2 gain-of-function mutations and the potential contribution of sterol uptake to Upc2-mediated azole resistance are under investigation.

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REFERENCES

1. Clinical and Laboratory Standards Institute. 2008. Reference method for broth dilution antifungal susceptibility testing of yeasts; approved method M27–A3. Clinical and Laboratory Standards Institute, Wayne, PA.
2. Coste AT, et al. 2006. A mutation in Tac1p, a transcription factor regulating CDR1 and CDR2, is coupled with loss of heterozygosity at chromosome 5 to mediate antifungal resistance in Candida albicans. Genetics 172:2139–2156.
3. Coste AT, Karababa M, Ischer F, Bille J, Sanglard D. 2004. Tac1, transcriptional activator of CDR genes, is a new transcription factor involved in the regulation of Candida albicans ABC transporters CDR1 and CDR2. Eukaryot. Cell 3:1639–1652.
4. Crowley JH, Leak FW, Jr, Shianna KV, Tove S, Parks LW. 1998. A mutation in a purported regulatory gene affects control of sterol uptake in Saccharomyces cerevisiae. J. Bacteriol. 180:4177–4183.
5. Davies BS, Rine J. 2006. A role for sterol levels in oxygen sensing in Saccharomyces cerevisiae. Genetics 174:191–201.
6. Dunkel N, Blass J, Rogers PD, Morschhauser J. 2008. Mutations in the multi-drug resistance regulator MRR1, followed by loss of heterozygosity, are the main cause of MDR1 overexpression in fluconazole-resistant Candida albicans strains. Mol. Microbiol. 69:827–840.
7. Dunkel N, et al. 2008. A gain-of-function mutation in the transcription factor Upc2p causes upregulation of ergosterol biosynthesis genes and increased fluconazole resistance in a clinical Candida albicans isolate. Eukaryot. Cell 7:1180–1190.
8. Reference deleted.
9. Feigal DW, et al. 1991. The prevalence of oral lesions in HIV-infected homosexual and bisexual men: three San Francisco epidemiological cohorts. AIDS 5:519–525.
10. Franz R, Ruhnke M, Morschhauser J. 1999. Molecular aspects of fluconazole resistance development in Candida albicans. Mycoses 42:453–458.
11. Heilmann CJ, Schneider S, Barker KS, Rogers PD, Morschhauser J. 2010. An A643T mutation in the transcription factor Upc2p causes constitutive ERG11 upregulation and increased fluconazole resistance in Candida albicans. Antimicrob. Agents Chemother. 54:353–359.
12. Hoot SJ, Smith AR, Brown RP, White TC. 2011. An A643V amino acid substitution in Upc2p contributes to azole resistance in well-characterized clinical isolates of Candida albicans. Antimicrob. Agents Chemother. 55:940–942.
13. Klein RS, et al. 1984. Oral candidiasis in high-risk patients as the initial manifestation of the acquired immunodeficiency syndrome. N. Engl. J. Med. 311:354–358.

14. Kontoyiannis DP, Lewis RE. 2002. Antifungal drug resistance of pathogenic fungi. Lancet 359:1135–1144.

15. Reference deleted.

16. Lin HX, White KA. 2004. A complex network of RNA-RNA interactions controls subgenomic miRNA transcription in a tombusvirus. EMBO J. 23:3365–3374.

17. Lopez-Ribot JL, et al. 1998. Distinct patterns of gene expression associated with development of fluconazole resistance in serial candida albicans isolates from human immunodeficiency virus-infected patients with oropharyngeal candidiasis. Antimicrob. Agents Chemother. 42:2932–2937.

18. MacPherson S, Larochelle M, Turcotte B. 2006. A fungal family of transcriptional regulators: the zinc cluster proteins. Microbiol. Mol. Biol. Rev. 70:583–604.

19. MacPherson S, et al. 2005. Candida albicans zinc cluster protein Upc2p confers resistance to antifungal drugs and is an activator of ergosterol biosynthetic genes. Antimicrob. Agents Chemother. 49:1745–1752.

20. Marie C, Leyde S, White TC. 2008. Cytoplasmic localization of sterol transcription factors Upc2p and Ecm22p in S. cerevisiae. Fungal Genet. Biol. 45:1430–1438.

21. Morschhauser J. 2002. The genetic basis of fluconazole resistance development in Candida albicans. Biochim. Biophys. Acta 1587:240–248.

22. Morschhauser J, et al. 2007. The transcription factor Mrr1p controls expression of the MDR1 efflux pump and mediates multidrug resistance in Candida albicans. PLoS Pathog. 3:e164. doi:10.1371/journal.ppat.0030164.

23. Perea S, et al. 2001. Prevalence of molecular mechanisms of resistance to azole antifungal agents in Candida albicans strains displaying high-level fluconazole resistance isolated from human immunodeficiency virus-infected patients. Antimicrob. Agents Chemother. 45:2676–2684.

24. Rangel-Frausto MS, et al. 1999. National epidemiology of mycoses survey (NEMIS): variations in rates of bloodstream infections due to Candida species in seven surgical intensive care units and six neonatal intensive care units. Clin. Infect. Dis. 29:253–258.

25. Reuss O, Vik A, Kolter R, Morschhauser J. 2004. The SAT1 flipper, an optimized tool for gene disruption in Candida albicans. Gene 341:119–127.

26. Rex JH, Rinaldi MG, Pfaffer MA. 1995. Resistance of Candida species to fluconazole. Antimicrob. Agents Chemother. 39:1–8.

27. Schmitt ME, Brown TA, Trumpower BL. 1990. A rapid and simple method for preparation of RNA from Saccharomyces cerevisiae. Nucleic Acids Res. 18:3091–3092.

28. Schubert S, et al. 2011. Regulation of efflux pump expression and drug resistance by the transcription factors Mrr1p, Upc2p, and Cap1 in Candida albicans. Antimicrob. Agents Chemother. 55:2212–2223.

29. Selmecki A, Forche A, Berman J. 2006. Aneuploidy and isochromosome formation in drug-resistant Candida albicans. Science 313:367–370.

30. Silver PM, Oliver BG, White TC. 2004. Role of Candida albicans transcription factor Upc2p in drug resistance and sterol metabolism. Fucar. Cell 3:1391–1397.

31. Warrillow AG, et al. 2010. Azole binding properties of Candida albicans sterol 14-alpha demethylase (CaCYP51). Antimicrob. Agents Chemother. 54:4235–4245.

32. White TC, Marr KA, Bowden RA. 1998. Clinical, cellular, and molecular factors that contribute to antifungal drug resistance. Clin. Microbiol. Rev. 11:382–402.

33. Znaidi S, et al. 2008. Genomewide location analysis of Candida albicans Upc2p, a regulator of sterol metabolism and azole drug resistance. Fucar. Cell 7:836–847.