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# An A643T Mutation in the Transcription Factor Upc2p Causes Constitutive *ERG11* Upregulation and Increased Fluconazole Resistance in *Candida albicans*▽

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**The zinc cluster transcription factor Upc2p mediates upregulation of ergosterol biosynthesis genes in response to ergosterol depletion in the fungal pathogen *Candida albicans*. One mechanism of acquired resistance to the antifungal drug fluconazole, which inhibits ergosterol biosynthesis, is constitutively increased expression of the *ERG11* gene encoding the drug target enzyme. A G648D mutation in Upc2p has recently been shown to cause hyperactivity of the transcription factor, resulting in overexpression of ergosterol biosynthesis genes and increased fluconazole resistance. In order to investigate if gain-of-function mutations in Upc2p are a common mechanism of *ERG11* upregulation and fluconazole resistance, we sequenced the *UPC2* alleles of four *ERG11*-overexpressing, fluconazole-resistant *C. albicans* isolates and matched susceptible isolates from the same patients. In three of the isolate pairs, no differences in the *UPC2* alleles were found, suggesting that mechanisms other than Upc2p mutations can cause *ERG11* overexpression. One resistant isolate had become homozygous for a *UPC2* allele containing a G1927A substitution that caused an alanine-to-threonine exchange at amino acid position 643 of Upc2p. Replacement of one of the endogenous *UPC2* alleles in a fluconazole-susceptible strain by the *UPC2*$^{A643T}$ allele resulted in *ERG11* overexpression and increased fluconazole resistance, which was further elevated when the A643T mutation was also introduced into the second *UPC2* allele. These results further establish gain-of-function mutations in *UPC2*, which can be followed by loss of heterozygosity for the mutated allele, as a mechanism of *ERG11* overexpression and increased fluconazole resistance in *C. albicans*, but other mechanisms of *ERG11* upregulation also exist.**

The antimycotic agent fluconazole, which inhibits ergosterol biosynthesis, is used widely to treat infections by the fungal pathogen *Candida albicans*. *C. albicans* can develop resistance to fluconazole by various mechanisms, including mutations in the *ERG11* gene encoding the drug target enzyme, overexpression of *ERG11*, and upregulation of efflux pumps that transport the drug out of the cells. Often, several of these mechanisms are combined to result in high-level fluconazole resistance and therapy failure (for a review, see reference 11).

In recent years, transcription factors that regulate the expression of genes mediating fluconazole resistance in *C. albicans* have been identified. Tac1p (*t*ranscriptional *a*ctivator of *CDR* genes) controls expression of the ATP-binding cassette (ABC) transporters *CDR1* and *CDR2*, whereas Mrr1p (*m*ultidrug *r*esistance *r*egulator) regulates expression of the *MDR1* gene encoding an efflux pump of the major facilitator superfamily (3, 12). Gain-of-function mutations in these transcription factors result in constitutive upregulation of their target genes and increased drug resistance (1, 2, 4, 12, 22). Upc2p, which like Tac1p and Mrr1p belongs to the zinc cluster transcription factor family that is specific for fungi, regulates the expression of *ERG11* and other ergosterol biosynthesis genes in response to ergosterol depletion (8, 10, 13, 20, 23). Recently, a G1943A mutation, which causes an amino acid substitution at position 648 in Upc2p from glycine to aspartate (G648D), was found in one of the two *UPC2* alleles of a fluconazole-resistant *C. albicans* isolate (S2) that exhibited increased expression of *ERG11* and other *ERG* genes as well as of *UPC2* itself compared to a matched, fluconazole-susceptible isolate (S1) from the same patient (5). Introduction of the *UPC2*$^{G648D}$ allele into the fluconazole-susceptible *C. albicans* strain SC5314 resulted in constitutive upregulation of *ERG11* and other Upc2p target genes and increased fluconazole resistance, demonstrating that gain-of-function mutations in this transcription factor can contribute to the development of drug resistance in *C. albicans* (5).

While mutations in Tac1p and Mrr1p are the cause of overexpression of *CDR1/2* and *MDR1*, respectively, in all fluconazole-resistant *C. albicans* isolates investigated to date, the G648D mutation is the only example of an activating mutation in *UPC2* causing *ERG11* upregulation in a clinical *C. albicans* isolate so far. *ERG11* overexpression has been observed with other fluconazole-resistant *C. albicans* isolates, but the mechanism of this upregulation remained unknown (6, 14, 21). Therefore, we set out to determine if mutations in *UPC2* might be a common mechanism of *ERG11* overexpression in *C. albicans*.

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TABLE 1. *C. albicans* strains used in this study

| Strain | Parent | Relevant characteristics or genotype[a] | Reference or source |
|---|---|---|---|
| Clinical isolates | | | |
| 1002 | | $FLU^S$ isolate from patient 9 | 14 |
| 3795 | | $FLU^R$ isolate from patient 9 | 14 |
| 580 | | $FLU^S$ isolate from patient 14 | 14 |
| 2440 | | $FLU^R$ isolate from patient 14 | 14 |
| 945 | | $FLU^S$ isolate from patient 15 | 14 |
| 1619 | | $FLU^R$ isolate from patient 15 | 14 |
| 5044 | | $FLU^S$ isolate from patient 28 | 14 |
| 5052 | | $FLU^R$ isolate from patient 28 | 14 |
| SC5314 | | Wild-type reference strain | 7 |
| Strains carrying introduced *UPC2* alleles | | | |
| SCUPC2R11A and -B | SC5314 | *UPC2*$^{G648D}$*-SAT1-FLIP/UPC2-2* | This study |
| SCUPC2R12A | SCUPC2R11A | *UPC2*$^{G648D}$*-FRT/UPC2-2* | This study |
| SCUPC2R12B | SCUPC2R11B | *UPC2*$^{G648D}$*-FRT/UPC2-2* | This study |
| SCUPC2R13A | SCUPC2R12A | *UPC2*$^{G648D}$*-FRT/UPC2*$^{G648D}$*-SAT1-FLIP* | This study |
| SCUPC2R13B | SCUPC2R12B | *UPC2*$^{G648D}$*-FRT/UPC2*$^{G648D}$*-SAT1-FLIP* | This study |
| SCUPC2R14A | SCUPC2R13A | *UPC2*$^{G648D}$*-FRT/UPC2*$^{G648D}$*-FRT* | This study |
| SCUPC2R14B | SCUPC2R13B | *UPC2*$^{G648D}$*-FRT/UPC2*$^{G648D}$*-FRT* | This study |
| SCUPC2R21A and -B | SC5314 | *UPC2-SAT1-FLIP/UPC2-2* | This study |
| SCUPC2R22A | SCUPC2R21A | *UPC2-FRT/UPC2-2* | This study |
| SCUPC2R22B | SCUPC2R21B | *UPC2-FRT/UPC2-2* | This study |
| SCUPC2R23A | SCUPC2R22A | *UPC2-FRT/UPC2-SAT1-FLIP* | This study |
| SCUPC2R23B | SCUPC2R22B | *UPC2-FRT/UPC2-SAT1-FLIP* | This study |
| SCUPC2R24A | SCUPC2R23A | *UPC2-FRT/UPC2-FRT* | This study |
| SCUPC2R24B | SCUPC2R23B | *UPC2-FRT/UPC2-FRT* | This study |
| SCUPC2R31A and -B | SC5314 | *UPC2*$^{A643T}$*-SAT1-FLIP/UPC2-2* | This study |
| SCUPC2R32A | SCUPC2R31A | *UPC2*$^{A643T}$*-FRT/UPC2-2* | This study |
| SCUPC2R32B | SCUPC2R31B | *UPC2*$^{A643T}$*-FRT/UPC2-2* | This study |
| SCUPC2R33A | SCUPC2R32A | *UPC2*$^{A643T}$*-FRT/UPC2*$^{A643T}$*-SAT1-FLIP* | This study |
| SCUPC2R33B | SCUPC2R32B | *UPC2*$^{A643T}$*-FRT/UPC2*$^{A643T}$*-SAT1-FLIP* | This study |
| SCUPC2R34A | SCUPC2R33A | *UPC2*$^{A643T}$*-FRT/UPC2*$^{A643T}$*-FRT* | This study |
| SCUPC2R34B | SCUPC2R33B | *UPC2*$^{A643T}$*-FRT/UPC2*$^{A643T}$*-FRT* | This study |
| Reporter strains expressing *GFP* from the *ERG11* promoter | | | |
| SCEG2A and -B | SC5314 | *UPC2-1/UPC2-2* *ERG11-1/erg11-2*::$P_{ERG11}$*-GFP-caSAT1* | This study |
| SCUPC2R12EG2A | SCUPC2R12A | *UPC2*$^{G648D}$*-FRT/UPC2-2* *ERG11-1/erg11-2*::$P_{ERG11}$*-GFP-caSAT1* | This study |
| SCUPC2R12EG2B | SCUPC2R12B | *UPC2*$^{G648D}$*-FRT/UPC2-2* *erg11-1*::$P_{ERG11}$*-GFP-caSAT1/ERG11-2* | This study |
| SCUPC2R14EG2A | SCUPC2R14A | *UPC2*$^{G648D}$*-FRT/UPC2*$^{G648D}$*-FRT* *ERG11-1/erg11-2*::$P_{ERG11}$*-GFP-caSAT1* | This study |
| SCUPC2R14EG2B | SCUPC2R14B | *UPC2*$^{G648D}$*-FRT/ UPC2*$^{G648D}$*-FRT* *erg11-1*::$P_{ERG11}$*-GFP-caSAT1/ERG11-2* | This study |
| SCUPC2R22EG2A | SCUPC2R22A | *UPC2-FRT/UPC2-2* *erg11-1*::$P_{ERG11}$*-GFP-caSAT1/ERG11-2* | This study |
| SCUPC2R22EG2B | SCUPC2R22B | *UPC2-FRT/UPC2-2* *ERG11-1/erg11-2*::$P_{ERG11}$*-GFP-caSAT1* | This study |
| SCUPC2R24EG2A | SCUPC2R24A | *UPC2-FRT/UPC2-FRT* *ERG11-1/erg11-2*::$P_{ERG11}$*-GFP-caSAT1* | This study |
| SCUPC2R24EG2B | SCUPC2R24B | *UPC2-FRT/ UPC2-FRT* *erg11-1*::$P_{ERG11}$*-GFP-caSAT1/ERG11-2* | This study |
| SCUPC2R32EG2A | SCUPC2R32A | *UPC2*$^{A643T}$*-FRT/UPC2-2* *erg11-1*::$P_{ERG11}$*-GFP-caSAT1/ERG11-2* | This study |
| SCUPC2R32EG2B | SCUPC2R32B | *UPC2*$^{A643T}$*-FRT/UPC2-2* *ERG11-1/erg11-2*::$P_{ERG11}$*-GFP-caSAT1* | This study |
| SCUPC2R34EG2A | SCUPC2R34A | *UPC2*$^{A643T}$*-FRT/UPC2*$^{A643T}$*-FRT* *ERG11-1/erg11-2*::$P_{ERG11}$*-GFP-caSAT1* | This study |
| SCUPC2R34EG2B | SCUPC2R34B | *UPC2*$^{A643T}$*-FRT/ UPC2*$^{A643T}$*-FRT* *erg11-1*::$P_{ERG11}$*-GFP-caSAT1/ERG11-2* | This study |

[a] *SAT1-FLIP* denotes the *SAT1* flipper cassette. The *UPC2* and *ERG11* alleles in strain SC5314 can be distinguished by EcoRI and HindIII restriction site polymorphisms, respectively. The *UPC2* allele containing the polymorphic EcoRI site at position +1593 was arbitrarily designated *UPC2-2* (see Table 4), and the *ERG11* allele containing the polymorphic HindIII site at position +347 was designated *ERG2-2*.

TABLE 2. Primers used in this study[a]

| Primer | Sequence |
|---|---|
| ACT19 | 5′-ATATA<u>CCGCGG</u>ACATTTTATGATGGAATGA-3′ |
| ERG13 | 5′-CGACATTATTA<u>GGGCCC</u>TTTGAGAACAGCC-3′ |
| ERG14 | 5′-CAATAGCCATATT<u>GTCGAC</u>TGATCTTCTTG-3′ |
| ERG15 | 5′-AATA<u>CTGCAG</u>CAACTTTCTTTCGATTCAGTG-3′ |
| ERG16 | 5′-CTAA<u>GAGCTC</u>GAATCCTGGTCCTATATTAGC-3′ |
| UPC2-3A | 5′-AACA<u>GAGCTC</u>TACGTTATTCAGCTTTCC-3′ |
| UPC2-3B | 5′-GCTTCATTAGCACAGTTGCCCATC-3′ |
| UPC2-4A | 5′-TTAT<u>GGGCCC</u>ACAGTAACGAATCACATTTGTG-3′ |
| UPC2-4B | 5′-GCATTCAATACTTGCCTTTAGTGC-3′ |
| ERG11-f | 5′-TTTAGTTTCTCCAGGTTATGCTCAT-3′ |
| ERG11-r | 5′-ATTAGCTTTGGCAGCAGCAGTA-3′ |
| UPC2-f | 5′-TCCATCCTTGACCCCTAGTCCT-3′ |
| UPC2-r | 5′-CGGCTGAGTTTTGATGTCTTGA-3′ |
| 18S-f | 5′-CACGACGGAGTTTCACAAGA-3′ |
| 18S-r | 5′-CGATGGAAGTTTGAGGCAAT-3′ |

[a] Restriction sites introduced into the primers are underlined. Primers UPC2-3B and UPC2-4B were used for sequencing *UPC2* internal regions. The primer pairs ERG11-f and ERG11-r, UPC2-f and UPC2-r, and 18S-f and 18S-r were used for quantitative real-time PCR and yielded 135-bp, 100-bp, and 52-bp products, respectively.

## MATERIALS AND METHODS

**Strains and growth conditions.** The *C. albicans* strains used in this study are listed in Table 1. All strains were stored as frozen stocks with 15% glycerol at −80°C and subcultured on YPD agar plates (10 g yeast extract, 20 g peptone, 20 g glucose, 15 g agar per liter) at 30°C. For routine growth of the strains, YPD liquid medium was used. For selection of nourseothricin-resistant transformants, 200 μg/ml nourseothricin (Werner Bioagents, Jena, Germany) was added to YPD agar plates. To obtain nourseothricin-sensitive derivatives in which the *SAT1* flipper cassette was excised by FLP-mediated recombination, transformants were grown overnight in YPM medium (10 g yeast extract, 20 g peptone, 20 g maltose per liter) without selective pressure to induce the *MAL2* promoter that controls expression of the *caFLP* gene in the *SAT1* flipper cassette. Cells (100 to 200) were then spread on YPD plates containing 20 μg/ml nourseothricin and grown for 2 days at 30°C. Nourseothricin-sensitive clones were identified by their small colony size and confirmed by restreaking on YPD plates containing 100 μg/ml nourseothricin as described previously (17).

**Quantitative real-time RT-PCR.** Total RNA was isolated from the clinical *C. albicans* isolates as previously described (5). An aliquot of the RNA preparations was used for quantitative real-time reverse transcription (RT)-PCR studies. First-strand cDNAs were synthesized from 2 μg of total RNA in a 21-μl reaction volume by using a SuperScript first-strand synthesis system for RT-PCR (Invitrogen) in accordance with the manufacturer's instructions. Quantitative real-time PCRs were performed in triplicate using a 7000 sequence detection system (Applied Biosystems, Inc., Foster City, CA). Independent PCRs were performed using the same cDNA for both the genes of interest and the 18S rRNA gene with SYBR green PCR master mix (Applied Biosystems, Inc.). Gene-specific primers were designed for the gene of interest and the 18S rRNA gene by using Primer Express software (Applied Biosystems, Inc.) and an Oligo analysis and plotting tool (Qiagen, Valencia, CA) and are shown in Table 2. The PCR conditions consisted of AmpliTaq Gold activation at 95°C for 10 min, followed by 40 cycles of denaturation at 95°C for 15 s and annealing/extension at 60°C for 1 min. A dissociation curve was generated at the end of each PCR cycle, using software provided with the 7000 sequence detection system, to verify that a single product was amplified. The change in fluorescence of SYBR green I dye in every cycle was monitored by the system software, and the cycle threshold ($C_T$) above the background for each reaction was calculated. The $C_T$ value of the 18S rRNA gene was subtracted from that of the gene of interest to obtain a $\Delta C_T$ value. The $\Delta C_T$ value of a calibrator (e.g., an azole-susceptible isolate sample of each matched set) was subtracted from the $\Delta C_T$ value of each sample to obtain a $\Delta\Delta C_T$ value. The gene expression level relative to the calibrator (change in expression) was expressed as $2^{-\Delta\Delta C_T}$. The $\Delta C_T$ values were also used to calculate standard error values.

TABLE 3. Relative *ERG11* and *UPC2* transcript levels in FLU$^S$ and FLU$^R$ clinical isolate pairs[c]

| Strain[a] | MIC$_{FLU}$ (μg/ml)[b] | *ERG11* | *UPC2* |
|---|---|---|---|
| 1002 (S) | 0.25 | 1 | 1 |
| 3795 (R) | >64 | 6.4 (±1.2) | 2.0 (±0.6) |
| 580 (S) | 1 | 1 | 1 |
| 2440 (R) | 32 | 4.0 (±0.1) | 5.5 (±0.2) |
| 945 (S) | 4 | 1 | 1 |
| 1619 (R) | 32 | 3.7 (±0.3) | 9.6 (±0.3) |
| 5044 (S) | 4 | 1 | 1 |
| 5052 (R) | 32 | 1.9 (±0.3) | 2.7 (±0.1) |

[a] Matched fluconazole-susceptible (S) and -resistant (R) isolates are grouped together.
[b] MIC data are from reference 14 (24-h readings).
[c] *ERG11* and *UPC2* mRNA levels of the susceptible isolates were set as 1.

**Plasmid constructions.** The *UPC2* alleles of the clinical *C. albicans* isolates were amplified with the primers UPC2-3A and UPC2-4A, which bind in the upstream and downstream region, respectively, of the *UPC2* gene (all primers used in this study are listed in Table 2). The PCR products were digested at the introduced SacI and ApaI sites and cloned into the vector pBluescript KSII, generating plasmids pUPC2-580-1, pUPC2-580-2, pUPC2-2440-1, pUPC2-2440-2, pUPC2-945-1, pUPC2-945-2, pUPC2-1619-1, pUPC2-1619-2, pUPC2-1002-1, pUPC2-1002-2, pUPC2-3795-1, pUPC2-3795-2, pUPC2-5044-1, pUPC2-5044-2, and pUPC2-5052 (a plasmid's name reflects the cloned *UPC2* allele and the *C. albicans* isolate from which it was obtained).

Plasmids pUPC2R1, pUPC2R2, and pUPC2R3, which were used to sequentially replace the resident *UPC2* alleles in strain SC5314 by mutated *UPC2* alleles or a nonmutated control construct with the help of the *SAT1* flipper cassette (see Fig. 1), were generated as follows. pUPC2R1 was generated by amplifying the *UPC2*$^{G648D}$-$T_{ACT1}$ fragment from plasmid pUPC2K3 (5) with the primers UPC2-3A and ACT19 and substituting the SacI/SacII-digested PCR product for the *UPC2* upstream region in the previously described plasmid pUPC2M2 (5). To obtain pUPC2R2, a SacI-BglII fragment with the wild-type *UPC2* allele from plasmid pUPC2K2 (5) was substituted for the corresponding fragment in pUPC2R1. pUPC2R3 was constructed by substituting an Nco-NdeI fragment containing the G1927A mutation from pUPC2-5052 for the corresponding fragment in pUPC2R1.

A $P_{ERG11}$-*GFP* reporter fusion was generated by amplifying an *ERG11* upstream fragment from genomic DNA of strain SC5314 with the primers ERG13 and ERG14 and substituting the ApaI/SalI-digested PCR product for the *OPT1* upstream fragment in plasmid pOPT1G22 (16) to obtain pERG11G1. The *ERG11* downstream region was then amplified with the primers ERG15 and ERG16 and the PstI/SacI-digested PCR product used to replace the *OPT1* downstream region in pERG11G1, resulting in pERG11G2 (see Fig. 3).

***C. albicans* transformation.** *C. albicans* strains were transformed by electroporation (9) with the gel-purified inserts from plasmids pUPC2R1, pUPC2R2, pUPC2R3, and pERG11G2. Nourseothricin-resistant transformants were selected as described previously (17), and single-copy integration of all constructs was confirmed by Southern hybridization with the probes shown in Fig. 1 and 3.

**Isolation of genomic DNA and Southern hybridization.** Genomic DNA from *C. albicans* strains was isolated as described previously (17). The DNA was digested with appropriate restriction enzymes, separated on a 1% agarose gel, and after ethidium bromide staining, 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 (GE Healthcare, Braunschweig, Germany) according to the instructions of the manufacturer.

**Drug susceptibility tests.** Fluconazole susceptibility tests were carried out in high-resolution medium (14.67 g HR medium [Oxoid GmbH, Wesel, Germany], 1 g $NaHCO_3$, 0.2 M phosphate buffer [pH 7.2]) using a previously described microdilution method (18). Readings were done after 24 h.

**FACS analysis.** Fluorescence-activated cell sorter (FACS) analysis was performed with a FACSCalibur cytometry system equipped with an argon laser emitting at 488 nm (Becton Dickinson, Heidelberg, Germany). Fluorescence was

TABLE 4. Polymorphisms in *UPC2* alleles of different *C. albicans* strains

| Strain | Allele | Nucleotide substitutions[a] |
|---|---|---|
| SC5314 (S) | 1 | T1338C, C1392T, C1410A, C1539T |
| | 2 | A1203G, A1593G |
| 1002 (S) | 1 | T1338C, C1392T, C1410A, C1539T |
| | 2 | A1203G, A1593G |
| 3795 (R) | 1 | T1338C, C1392T, C1410A, C1539T |
| | 2 | A1203G, A1593G |
| 580 (S) | 1 | T777A, T879C, C1326T, T1338C, C1392T, C1410A, C1539T |
| | 2 | A234G, A276G, T387C, **T425G**, T747A, T777A, T879C, A1203G, A1593G |
| 2440 (R) | 1 | T777A, T879C, C1326T, T1338C, C1392T, C1410A, C1539T |
| | 2 | A234G, A276G, T387C, **T425G**, T747A, T777A, T879C, A1203G, A1593G |
| 945 (S) | 1 | A1203G, A1593G |
| | 2 | A234G, A276G, T387C, **T425G**, T747G, T777A, T879C, A1203G, A1593G |
| 1619 (R) | 1 | A1203G, A1593G |
| | 2 | A234G, A276G, T387C, **T425G**, T747G, T777A, T879C, A1203G, A1593G |
| 5044 (S) | 1 | T777A, T879C, C1326T, T1338C, C1392T, C1410A, C1539T |
| | 2 | A234G, A276G, T387C, **T425G**, T747A, T777A, T879C, A1203G, A1593G, **G1927A** |
| 5052 (R) | | A234G, A276G, T387C, **T425G**, T747A, T777A, T879C, A1203G, A1593G, **G1927A** |

[a] orf19.391 in the *Candida* genome database (http://www.candidagenome.org/) was used as the reference sequence. The A1593G substitution (underlined) creates an EcoRI restriction site that was used to distinguish the two *UPC2* alleles in strain SC5314. The sequences of the two *UPC2* alleles of strain SC5314 were obtained from cloned copies (reference 15 and unpublished data). Nucleotide substitutions that result in amino acid exchanges in Upc2p are highlighted in bold. The T425G substitution, which was found in several *UPC2* alleles from $FLU^S$ and $FLU^R$ isolates, causes the amino acid exchange I142S. The G1927A substitution results in the A643T gain-of-function mutation. Matched fluconazole-susceptible (S) and -resistant (R) isolates are grouped together.

measured on the FL1 fluorescence channel equipped with a 530-nm band-pass filter. Twenty thousand cells were analyzed per sample. Fluorescence data were collected by using logarithmic amplifiers. The mean fluorescence values were determined with CellQuest Pro (Becton Dickinson) software.

## RESULTS

**Sequence analysis of the *UPC2* alleles of *ERG11*-overexpressing *C. albicans* isolates.** To investigate if activating mutations in the transcription factor *UPC2* are a common cause of *ERG11* overexpression in fluconazole-resistant, clinical *C. albicans* isolates, we selected four pairs of fluconazole-susceptible and fluconazole-resistant isolates from HIV-infected patients in which the resistant isolate exhibited increased *ERG11* mRNA levels in Northern hybridization experiments (14). Quantitative RT-PCR confirmed that the resistant isolates 3795, 2440, 1619, and 5052 had increased *ERG11* transcript levels and also showed increased *UPC2* expression compared to the matched susceptible isolates 1002, 580, 945, and 5044, respectively (Table 3). We then cloned and sequenced the *UPC2* alleles of the eight isolates (see Materials and Methods; Table 4). All four susceptible isolates contained two polymorphic *UPC2* alleles. The resistant isolates 3795, 2440, and 1619 contained the same two alleles as their matched susceptible isolates, indicating that the increased *ERG11* expression in these isolates was not caused by mutations in the *UPC2* coding region. However, in isolate 5052 we found only one of the two *UPC2* alleles that were present in the matched susceptible isolate 5044, and direct sequencing of the amplified PCR products across a region that was polymorphic in isolate 5044 confirmed the loss of heterozygosity in isolate 5052. Interestingly, the *UPC2* allele that was retained in isolate 5052 contained a G1927A substitution which was not found in any of the other isolate pairs and resulted in an alanine-to-threonine exchange at position 643 (A643T) in the encoded protein. Although the susceptible isolate 5044 contained the same allele, the facts that the A643T mutation is located in the vicinity of the previously described G648D gain-of-function mutation in the C-terminal part of Upc2p and that the resistant isolate 5052 had become homozygous for the $UPC2^{A643T}$ allele suggested that this genomic alteration may have caused the increased *ERG11* and *UPC2* expression.

**The A643T mutation in Upc2p causes constitutive *ERG11* upregulation and increased fluconazole resistance.** To investigate whether the A643T mutation in Upc2p causes hyperactivity of the transcription factor and enhanced fluconazole resistance, we replaced one of the endogenous *UPC2* alleles of the fluconazole-susceptible *C. albicans* model strain SC5314 by the $UPC2^{A643T}$ allele with the help of the *SAT1* flipper cassette (17), as outlined in Fig. 1. This strategy allowed recycling of the *caSAT1* selection marker and subsequent introduction of the same mutation into the second *UPC2* allele to study the effect of loss of heterozygosity. For comparison, we also introduced the previously described G648D mutation (5) into one or both *UPC2* alleles of this strain in the same way. In addition, the resident *UPC2* alleles were also replaced in an identical fashion by a nonmutated wild-type *UPC2* allele to control for possible effects of the integration strategy. In each case, two independent series of strains were constructed to exclude unspecific phenotypic effects of the genetic manipulations. Introduction of the G1927A (A643T) and G1943A (G648D) mutations into the resident *UPC2* alleles was confirmed by reamplification of

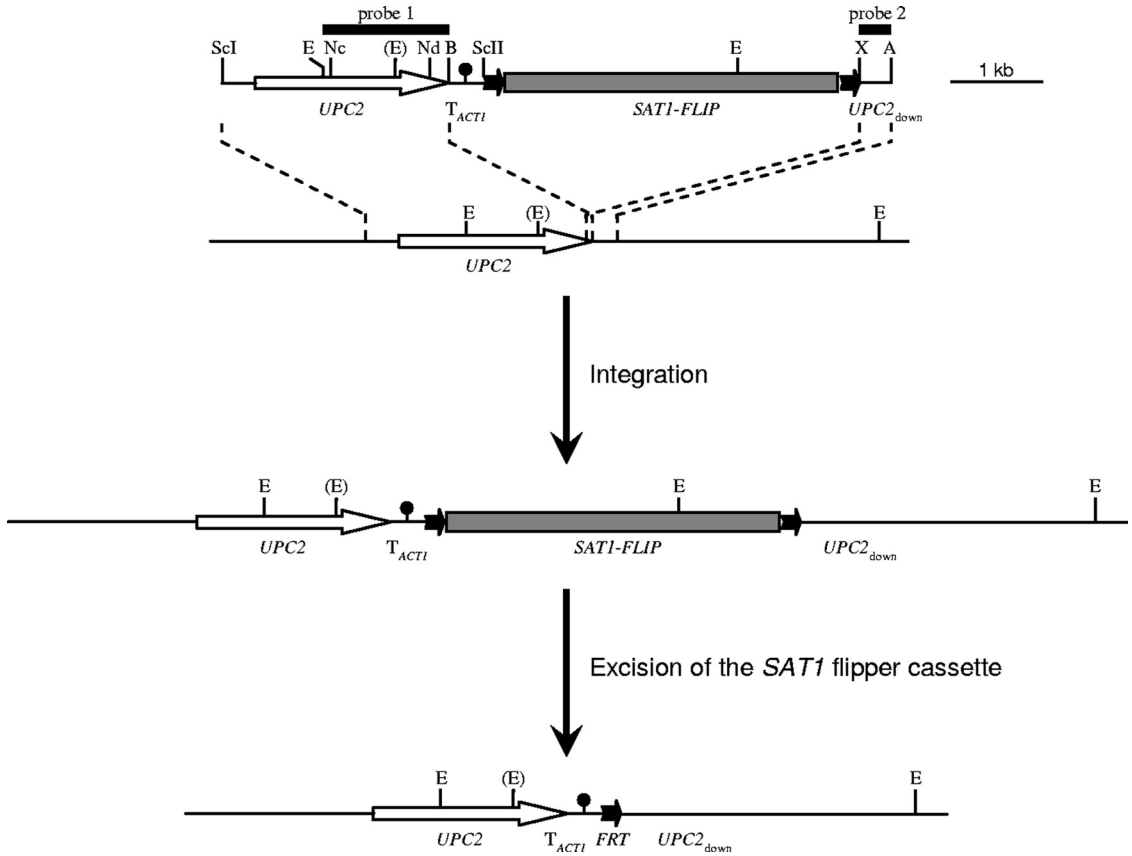


FIG. 1. Integration of different *UPC2* alleles into one or both endogenous *UPC2* loci of the *C. albicans* wild-type strain SC5314, followed by recycling of the *SAT1* flipper cassette (gray rectangle flanked by black arrows). The structure of the inserts from plasmids pUPC2R1, pUPC2R2, and pUPC2R3, which contain the *UPC2*$^{G648D}$ allele, the nonmutated wild-type *UPC2* allele, and the *UPC2*$^{A643T}$ allele, respectively, is shown on top. Relevant restriction sites are indicated as follows: A, ApaI; B, BglII; E, EcoRI; Nc, NcoI; Nd, NdeI; ScI, SacI; ScII, SacII; X, XhoI. The EcoRI site shown in parentheses is present only in the *UPC2-2* allele of strain SC5314 and in the *UPC2* allele contained in pUPC2R3. The black circle represents the transcription termination sequence of the *ACT1* gene ($T_{ACT1}$); the FLP recombination target sites (*FRT*) are symbolized by the black arrows. The EcoRI-BglII fragment (probe 1) and the XhoI-ApaI fragment (probe 2) from pUPC2R2 were used as probes in Southern hybridization experiments to confirm correct genomic insertion of the constructs and subsequent excision of the *SAT1* flipper cassette.

the *UPC2* alleles from heterozygous and homozygous mutants and sequencing of the PCR products.

As previously reported (5), replacement of one of the wild-type *UPC2* alleles by the *UPC2*$^{G648D}$ allele increased the MIC of fluconazole for the strains about fourfold (Fig. 2). Subsequent introduction of the G648D mutation into the second *UPC2* allele led to a further twofold increase in resistance, demonstrating that a change from heterozygosity to homozygosity for the mutated allele confers a selective advantage in the presence of the drug. Similarly, the introduction of the A643T mutation also conferred increased fluconazole resistance, albeit not as efficiently as the G648D mutation, with a stronger effect when the mutation was present in both alleles. Replacement of the resident *UPC2* alleles by a nonmutated, wild-type copy had no effect, confirming that the increased drug resistance was due to the G648D and A643T mutations.

As Upc2p is a transcription factor that regulates the expression of *ERG11* and other *ERG* genes, we investigated whether the resistance mutations in *UPC2* constitutively activated the *ERG11* promoter. For this purpose, we introduced a $P_{ERG11}$-*GFP* reporter fusion into the strains expressing the various *UPC2* alleles, such that *GFP* was expressed from one of the endogenous *ERG11* promoters (Fig. 3A). The basal activity of the *ERG11* promoter could be detected with *GFP* as a reporter gene, as transformants of the wild-type strain SC5314 carrying the reporter fusion exhibited fluorescence above the background of the nontransformed parental strain (Fig. 3B). In line with the fluconazole susceptibility tests, strains containing one copy of the mutated *UPC2*$^{G648D}$ or *UPC2*$^{A643T}$ alleles exhibited increased *ERG11* promoter activity, which was further elevated when the mutation was also present in the second *UPC2* allele. Again, the effect of the A643T mutation was less pronounced than that of the G648D mutation. In contrast, the control strains in which the resident *UPC2* alleles had been replaced by a nonmutated allele did not show increased *ERG11* promoter activity. These results demonstrate that both the G648D and A643T mutations in Upc2p result in hyperactivity of the transcription factor and constitutively elevated *ERG11* expression.

## DISCUSSION

In this work, we describe a second mutation in the transcription factor Upc2p in a fluconazole-resistant clinical *C. albicans*

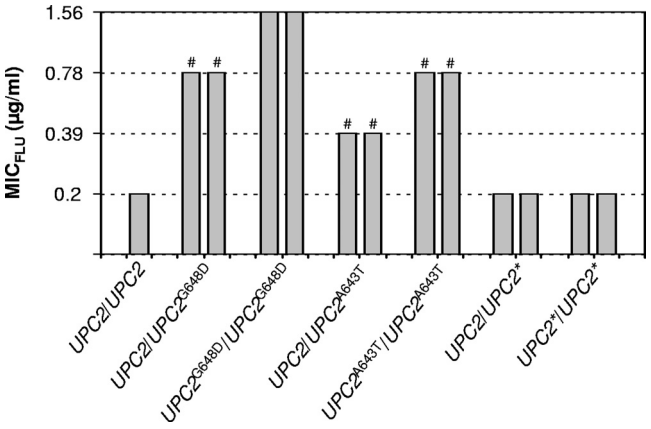


FIG. 2. Fluconazole susceptibilities of the wild-type parental strain SC5314 and derivatives in which one or both resident *UPC2* alleles were replaced by the *UPC2*$^{G648D}$ allele, the *UPC2*$^{A643T}$ allele, or a nonmutated wild-type *UPC2* allele (indicated by the asterisk). The results obtained with two independently generated strains (A and B) are shown in each case. The following strains were used (see Table 1): SC5314 (*UPC2*/*UPC2*), SCUPC2R12A and -B (*UPC2*/*UPC2*$^{G648D}$), SCUPC2R14A and -B (*UPC2*$^{G648D}$/*UPC2*$^{G648D}$), SCUPC2R32A and -B (*UPC2*/*UPC2*$^{A643T}$), SCUPC2R34A and -B (*UPC2*$^{A643T}$/*UPC2*$^{A643T}$), SCUPC2R22A and -B (*UPC2*/*UPC2**), and SCUPC2R24A and -B (*UPC2**/*UPC2**). #, reduced growth was observed at one dilution step below the MIC.

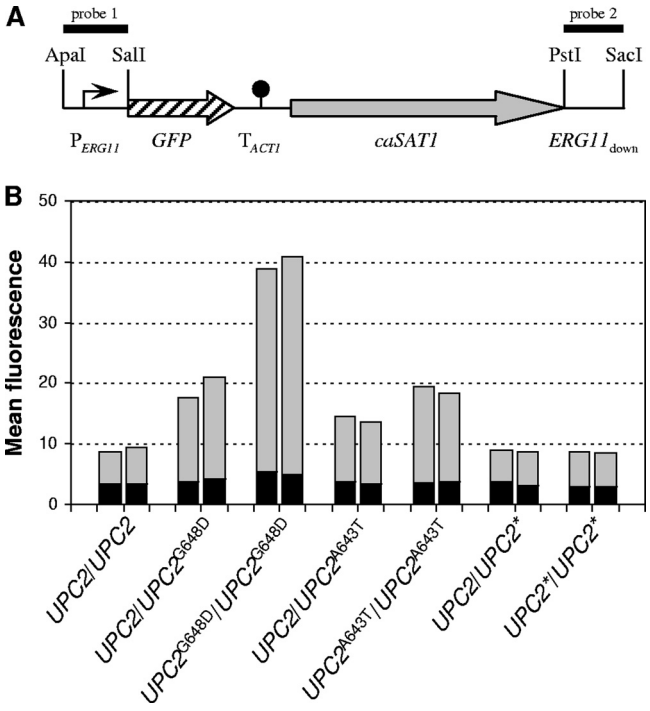


FIG. 3. The G648D and A643T mutations in Upc2p cause constitutive upregulation of the *ERG11* promoter. (A) Structure of the cassette from pERG11G2 containing the P$_{ERG11}$-*GFP* reporter fusion, which was integrated into one of the *ERG11* alleles of the various *C. albicans* strains by homologous recombination. The *ERG11* promoter (P$_{ERG11}$) is symbolized by a bent arrow, the *GFP* gene by a hatched arrow, the transcription termination sequence of the *ACT1* gene (T$_{ACT1}$) by the filled circle, and the *caSAT1* selection marker by the gray arrow. The ApaI-SalI fragment (probe 1) and the PstI-SacI fragment (probe 2) from pERG11G2 were used as probes in Southern hybridization analyses to confirm correct genomic integration. (B) Fluorescence of *C. albicans* strains carrying the P$_{ERG11}$-*GFP* reporter fusion in a wild-type background or mutant derivatives in which one or both endogenous *UPC2* alleles were replaced by the *UPC2*$^{G648D}$ allele, the *UPC2*$^{A643T}$ allele, or a nonmutated wild-type allele (*UPC2**). The strains were grown to log phase in YPD medium, and the mean fluorescence of the cells was determined by flow cytometry. The results obtained with two independently generated reporter strains are shown in each case. The following strains were used (see Table 1): SCEG2A and -B (*UPC2*/*UPC2*), SCUPC2R12EG2A and -B (*UPC2*/*UPC2*$^{G648D}$), SCUPC2R14EG2A and -B (*UPC2*$^{G648D}$/*UPC2*$^{G648D}$), SCUPC2R32EG2A and -B (*UPC2*/*UPC2*$^{A643T}$), SCUPC2R34EG2A and -B (*UPC2*$^{A643T}$/*UPC2*$^{A643T}$), SCUPC2R22EG2A and -B (*UPC2*/*UPC2**), and SCUPC2R24EG2A and -B (*UPC2**/*UPC2**). The background fluorescence of the parental strains, which do not contain the *GFP* gene, is indicated by the black part of each column.

isolate. Although the A643T substitution had a weaker effect than the G648D mutation that was previously found in another fluconazole-resistant isolate (5), it also resulted in constitutive upregulation of the *ERG11* promoter and increased fluconazole resistance when introduced into a drug-susceptible strain. These results confirm that gain-of-function mutations in Upc2p can be a cause of *ERG11* overexpression and contribute to fluconazole resistance in clinical *C. albicans* isolates. Interestingly, the G1927A mutation that results in the A643T substitution was already present in one of the two *UPC2* alleles of the susceptible isolate 5044. It can be hypothesized that the mutation was first acquired in a more susceptible progenitor of this isolate during fluconazole therapy, but isolates other than 5044 and 5052 were not available from this patient.

Our results also demonstrate that a change from heterozygosity to homozygosity for the *UPC2*$^{G648D}$ and *UPC2*$^{A643T}$ alleles further increases *ERG11* expression and fluconazole resistance. The previously described fluconazole-resistant isolate S2, which had acquired the G648D mutation, was heterozygous for the mutated *UPC2* allele, but continued exposure to the drug might have selected for loss of heterozygosity also in this strain. In contrast, isolate 5052 had become homozygous for the *UPC2*$^{A643T}$ allele. As the effect of the loss of heterozygosity was moderate when the mutated *UPC2* alleles were expressed in strain SC5314 (Fig. 2), it cannot account for the eightfold increase in fluconazole resistance of isolate 5052 compared to the matched isolate 5044. Isolate 5052 has been reported to overexpress the ABC transporters Cdr1 and Cdr2, which may contribute to its increased drug resistance (14). In addition, it was recently shown that isolate 5052 also had acquired a gain-of-function mutation in Mrr1p and become homozygous for the mutated *MRR1* allele (4). The Mrr1p mutation, which caused overexpression of the *MDR1* efflux pump, is likely to be responsible for part of the increase in fluconazole resistance in isolate 5052. As *UPC2* and *MRR1* are located on different chromosomes (chromosome 1 and chromosome 3, respectively), the two loss-of-heterozygosity events observed in isolate 5052 may have occurred independently.

Upc2p upregulates expression of *ERG11* and other ergosterol biosynthesis genes in response to ergosterol depletion, which occurs in the presence of azole drugs and other ergosterol biosynthesis inhibitors (10, 20). It is presently unknown how Upc2p is activated under these conditions. One model suggests that a transmembrane domain in the C-terminal region of the protein acts as a cytoplasmic anchor and that

proteolytic cleavage allows translocation of the transcription factor domain to the nucleus under inducing conditions, in analogy to the activation of mammalian sterol response element binding proteins (SREBPs) (20). On the other hand, Upc2p that was tagged at the C terminus with a 3× hemagglutinin (HA) epitope could be immunoprecipitated with an anti-HA antibody when bound to its target DNA (23). Therefore, at least the HA-tagged Upc2p, which was constitutively active, can bind to and activate its target genes without proteolytic processing. An alternative model suggests that inducing conditions may prevent Upc2p from interacting with a repressor or an intrinsic negative regulatory domain (23). Whatever the mechanism of Upc2p activation is, it is likely that the gain-of-function mutations in the C-terminal part of Upc2p mimic its activated state, resulting in upregulation of Upc2p target genes. Evidently, the hyperactivity of Upc2p caused by the gain-of-function mutations confers increased fluconazole resistance upon cells carrying these mutations.

In summary, this study demonstrates that gain-of-function mutations in the transcription factor Upc2p are a cause of *ERG11* overexpression and increased fluconazole resistance in *C. albicans*. Loss of heterozygosity for a hyperactive *UPC2* allele results in a further increase in drug resistance, similar to what has been found for strains carrying mutated *TAC1* and *MRR1* alleles (2, 4, 12). However, several *ERG11*-overexpressing isolates investigated in the present study did not exhibit mutations in the *UPC2* coding region, suggesting that other mechanisms can cause *ERG11* upregulation in fluconazole-resistant strains. As these isolates also showed increased *UPC2* expression, such mechanisms could include mutations in the *UPC2* promoter region or in upstream regulatory factors that control *UPC2* expression (8). Alternatively, gene amplification by whole-chromosome or segmental aneuploidy could also account for increased *ERG11* and *UPC2* expression (19).

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