SREBP-Dependent Triazole Susceptibility in Aspergillus fumigatus Is Mediated through Direct Transcriptional Regulation of erg11A (cyp51A)

Sara J. Blosser and Robert A. Cramer
Department of Immunology and Infectious Diseases, Montana State University, Bozeman, Montana, USA

As triazole antifungal drug resistance during invasive Aspergillus fumigatus infection has become more prevalent, the need to understand mechanisms of resistance in A. fumigatus has increased. The presence of two erg11 (cyp51) genes in Aspergillus spp. is hypothesized to account for the inherent resistance of this mold to the triazole fluconazole (FLC). Recently, an A. fumigatus null mutant of a transcriptional regulator in the sterol regulatory element binding protein (SREBP) family, the ΔsrbA strain, was found to have increased susceptibility to FLC and voriconazole (VCZ). In this study, we examined the mechanism engendering the observed increase in A. fumigatus triazole susceptibility in the absence of SrbA. We observed a significant reduction in the erg11A transcript in the ΔsrbA strain in response to FLC and VCZ. Transcript levels of erg11B were also reduced but not to the extent of erg11A. Interestingly, erg11A transcript levels increased upon extended VCZ, but not FLC, exposure. Construction of an erg11A conditional expression strain in the ΔsrbA strain was able to restore erg11A transcript levels and, consequently, wild-type MICs to the triazole FLC. The VCZ MIC was also partially restored upon increased erg11A transcript levels; however, total ergosterol levels remained significantly reduced compared to those of the wild type. Induction of the erg11A conditional strain did not restore the hypoxia growth defect of the ΔsrbA strain. Taken together, our results demonstrate a critical role for SrbA-mediated regulation of ergosterol biosynthesis and triazole drug interactions in A. fumigatus that may have clinical importance.

Aspergillus fumigatus is a human fungal pathogen that must adapt to a variety of in vivo obstacles in order to cause disease. Some of these significant obstacles include high temperature, macro- and micronutrient availability, pH variation, oxidative stress, and oxygen availability (5, 6, 10, 34, 55). Understanding the mechanisms employed by A. fumigatus to overcome these challenges may lead to the design of improved antifungal drugs and therapeutic options for patients. Current treatment for invasive aspergillosis (IA) typically involves utilization of the triazole class of antifungal drugs. While this class of drugs is not associated with significant toxicity issues of amphotericin B, some serious side effects have been reported and long-term usage is not ideal (24, 38, 40). In addition, resistance to the triazoles has recently been documented and appears to be becoming more prevalent in some areas of the world (14, 22, 51). Thus, understanding potential resistance mechanisms to these drugs is important for maintaining and improving our current standard of care.

Aspergillus spp. are inherently resistant to the triazole fluconazole (FLC), an antifungal drug commonly used in superficial and systemic Candida spp. and other fungal infections or prophylactically used prior to transplantation or cancer therapies (12, 33, 36, 41). This resistance precludes the use of this antifungal drug in the treatment of IA, though it is sometimes given prophylactically to patients at risk (56). The mechanism behind the inherent FLC resistance in Aspergillus spp. is thought to be due to the existence of multiple paralogs for the FLC target in the genome. Aspergillus spp. contain two genes encoding 14α-demethylase (erg11, also called cyp51), which is the target of the triazoles (36). Both of these isoforms have 40 to 70% homology with erg11 genes from other fungal species and ~60% homology to each other (36). Single gene replacement mutants of either erg11A or erg11B are viable (36). As observed in other fungi, complete elimination of erg11 in A. fumigatus by way of double gene replacement is lethal (23). Recently, FLC was shown to physically bind Erg11B with much greater efficiency than Erg11A, whereas VCZ was shown to physically bind both paralogs with high affinity (52). Together with the increased susceptibility of erg11A null mutants to FLC, these data suggest that the inherent FLC resistance by A. fumigatus is due largely to the two functional Erg11 enzymes (37). Along these lines, resistance to antimicrobial agents, including antifungal drugs, generally occurs via one of three mechanisms: target modification, target overexpression, or increased efflux of drug pumps (4). A fourth mechanism is observed in bacteria, namely, microbial degradation of the antimicrobial agent, but this mechanism has not been observed in fungi to date (4, 49). Transcription factor modification may be another understudied antifungal resistance mechanism and has been studied in the human fungal pathogen Candida albicans (8, 15).

In C. albicans, gain-of-function mutations in four transcription factors, Tac1, Mrr1, Cap1, and Upc2, have been linked to increased antifungal drug resistance (16, 17, 39, 44, 58). Gain-of-function mutations in Tac1, Mrr1, and Cap1 are associated with increased drug efflux and have been well characterized. Conversely, gain-of-function mutations in Upc2 do not confer increased expression of drug efflux pumps (44), and this resistance is thought to be due to an increase in the erg11 transcript or other Upc2 target genes (21, 44, 46, 54). Upc2 regulates sterol biosyn-
thesis and erg11 expression, demonstrating another mechanism whereby drug target overexpression can occur (17). However, whether such mutations occur in transcription factors or their binding sites in A. fumigatus is unknown. Recently, Albarraj et al. attributed the insertion of the AfI transposon into the erg11A/cyp51A promoter region of an A. fumigatus patient isolate to the azole resistance of that strain (1). However, the connection between this transposon insertion and the increased erg11A/cyp51A expression and azole drug resistance is currently unknown.

We have characterized a transcriptional regulator in the sterol regulatory element binding protein (SREBP) family, SrbA, in A. fumigatus that has an important role in hypoxia adaptation, polarized growth, virulence, and triazole drug interactions (55). The role of SREBPs in the regulation of ergosterol biosynthesis and hypoxia adaptation was originally observed in Schizosaccharomyces pombe and Cryptococcus neoformans (9, 11, 25, 50). In these fungi, SREBPs sense sterol levels as an indirect measure of oxygen availability, and the corresponding reduction of sterol levels in low oxygen conditions triggers N-terminal proteolytic cleavage of the endoplasmic reticulum (ER)-bound SREBP (25, 43). As the N terminus contains the transcription factor portion of this protein, translocation of the cleaved form triggers transcription of oxygen and sterol biosynthesis-related genes (18). Although direct binding between SREBPs and ergosterol biosynthesis genes has been demonstrated for erg3 (50), transcriptional profiling of SREBP mutants suggests that additional ergosterol biosynthesis genes are likely directly or indirectly regulated by SREBPs in fungi. These results indicate an important role for SREBPs in the regulation of ergosterol biosynthesis, much like the observed relationship between Upc2 and ergosterol biosynthesis in C. albicans (21, 44, 46, 54).

As ergosterol is the main sterol component of the cell membrane in most ascomycetes (53), and as this synthesis pathway and its resulting product is the target of both the polyene and triazole antifungal drugs, a better understanding of the transcriptional regulatory mechanisms of this pathway is clinically relevant. Importantly, transcriptome profiling of ΔsrbA in A. fumigatus has demonstrated differential regulation of erg3, erg25, and erg24, suggesting that regulation of sterol biosynthesis is also conserved in A. fumigatus (7a, 55). In this study, we observe that this differential gene expression, demonstrating another mechanism whereby drug target overexpression can occur (17). However, whether such mutations occur in transcription factors or their binding sites in A. fumigatus is unknown. Recently, Albarraj et al. attributed the insertion of the AfI transposon into the erg11A/cyp51A promoter region of an A. fumigatus patient isolate to the azole resistance of that strain (1). However, the connection between this transposon insertion and the increased erg11A/cyp51A expression and azole drug resistance is currently unknown.

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**MATERIALS AND METHODS**

**Strains and media.** Aspergillus fumigatus strain CEA17 was used to construct the srbA null mutant strain (55). The erg11A inducible strain was generated in a ΔsrbA strain in which the pyrG selectable marker was recycled. pyrG was recycled by excising a central 1.1-kb fragment of the pyrG open reading frame (ORF) from the srbA gene replacement plasmid and then transformed into the ΔsrbA background on media containing 5-fluoroorotic acid (Invitrogen). Confirmation of a single integration at the srbA locus was done with PCR and Southern blot analyses. For generation of the pnia-erg11A-ΔsrbA strain, genomic DNA was extracted from the pnia-erg11A-Δerg11B strain (23) (gift of Terry Roemer, Merck) and the pnia-erg11A construct was amplified via PCR, transformed into the pyrG-ΔsrbA strain, and screened as previously described (55). This strain was verified via Southern blot analysis for single, locus-specific integration of the pnia-erg11A construct. The wild-type strain referred to in this article is strain CBS 144.89 (gift of Jean-Paul Latgé, Institut Pasteur), which is the background strain for CEA17. All strains were routinely grown at 37°C on glucose minimal medium (GMM) that contains 1% glucose (45). For induction/repression experiments, strains were grown in liquid GMM with 20 mM sodium nitrate (NO₃⁻) or 20 mM ammonium tartrate (NH₄⁺) as the sole nitrogen source (23).

**Quantitative real-time PCR.** A. fumigatus strains were cultured in liquid media (GMM or induction/repression minimal media) for 12 h following germination of conidia, with the exception of the FLC and VCZ treatment experiments. Mycelia were harvested via vacuum filtration and lyophilized overnight prior to tissue disruption with 0.1 mm glass beads. Total RNA was extracted using TRIzol (Bioline) according to the manufacturer’s instructions and purified via RNeasy columns (Qiagen). Genomic DNA removal was completed with Turbo DNase I (Ambion). A secondary genomic DNA removal step was done with the Qiagen Quantitect reverse transcription kit (Qiagen). cDNA was synthesized via oligo(dT)-primed cDNA synthesis. This quantitative real-time PCR (qRT-PCR) was conducted on a Bio-Rad MyiQ5 real-time PCR detection system and analyzed using the IQ5 optical system software package, which utilizes the method pioneered by Livak and Schmittgen for quantitative analysis (29).

qRT-PCR was conducted in technical triplicates except where noted. The normalized fold expression graphed in each figure represents the means and standard deviations of three biological replicates as normalized to two housekeeping genes, such as tefA, GAPDH, or actin. An mRNA control was used as a no-template control in each analysis.

**FLC or VCZ treatment for qRT-PCR analysis was conducted by using the following steps.** (i) A. fumigatus strains were grown in liquid GMM at 37°C until germinated. (ii) Germings were treated with a 50% MIC of FLC (CBS 144.89, 128 μg/ml; ΔsrbA strain, 0.50 μg/ml) or VCZ (CBS 144.89, 0.0625 μg/ml; ΔsrbA strain, 0.006 μg/ml) for each strain and solubilized in water or dimethyl sulfoxide (DMSO) for 6 and 12 h. (iii) Samples were then harvested, frozen, and lyophilized, and RNA was extracted as described above.

**Antifungal susceptibility testing.** Susceptibility to FLC and VCZ were evaluated with Etest strips (AB Biodisk). Strips were placed on an induction/repression agar plate containing a lawn of 10⁶ conidia. Growth inhibition was evaluated at 48 h by visualization of growth inhibition when grown at 37°C.

The standard RPMI-MOPS (where MOPS is morpholinopropanesulfonic acid) (Sigma-Aldrich) plates were not utilized due to the nitrogen source requirements of the strains utilized in this study.

**EMSA.** For SrbA protein preparation, cDNA encoding the first 275 amino acids of A. fumigatus SrbA were cloned into the plasmid pPAL7 to utilize the Proximity XAct tag (Bio-Rad). The resulting plasmid pPAL7_SrbA1-275 was transformed into Escherichia coli strain BL21(DE3). The recombinant protein was purified from E. coli using Proximity XAct purification resin columns according to the manufacturer’s instructions. (Bio-Rad). An affinity-purified rabbit polyclonal antibody was generated utilizing this recombinant protein by Pacific Immunology using standard protocols. The erg11A probe was amplified via PCR, and then biotin-labeled probes were allowed to anneal to the PCR template for 5 min at 95°C, followed by a 15-min incubation step at 37°C. Labeled probes were primers (IDT DNA) labeled with a 3’ biotin end biotinylation kit (Thermo Scientific) according to manufacturer’s instructions. A total of 40 fmol of the probe was coincubated with 1 μg of recombinant protein for 20 min at room temperature according to the
LightShift chemiluminescent electrophoretic mobility shift assay (EMSA) kit instructions (Thermo Scientific). This reaction mixture was run on a 6% native gel, transferred to a positively charged nylon membrane (Roche), and developed according to the chemiluminescent nucleic acid detection module instructions (Thermo Scientific). For the supershift assay, ~0.25 μg recombinant polyclonal SrbA antibody was coincubated with the above EMSA reaction mixture and developed as described previously.

**Total ergosterol extraction.** Total ergosterol from *A. fumigatus* strains was extracted 12 h postgermination in liquid GMM with 20 mM NO₃ (induced, ΔsrbA mutant and CBS 144.89) or liquid GMM with 20 mM NH₄ (repressed). Mycelia were harvested via vacuum filtration, dried, and weighed. Two hundred milligrams of dry fungal tissue was allowed to germinate in liquid culture and then dosed with a 50% MIC of FLC or VCZ for 6 and 12 h. Samples were incubated at 37°C for 1 h. Sterols were extracted with 1 ml of distilled water and 3 ml of pentane and vortexed for 3 min. The upper aqueous layer was allowed to separate from the cellular debris, and 1 ml of this extract was transferred to a clean glass tube and evaporated in a fume hood at room temperature. Upon preparation for injection, samples were resolved in 1 ml of methanol and syringe filtered through a 0.2 μm-pore-size filter (Acrodisc; Waters). Total ergosterol was measured using a Shimadzu CLASS-VP high-performance liquid chromatograph (HPLC) and detected via an SPD-M10AVP photo diode array at 280 nm on a μBondapack C₁₈ column (300 mm by 3.9 mm, 10 μm). The quantity of ergosterol per strain was calculated from a standard curve of ergosterol (Acros Organics, catalog no. 117801250).

**Sterol profile.** Mycelia were grown and harvested as described above for total ergosterol extraction. Extraction was done as described previously (3) and exactly as in the total ergosterol extraction, with the exception that the neutral lipids were extracted twice with 1.5 ml hexane. The upper aqueous layer was separated from the cellular debris, and 1 ml of this extract was evaporated via nitrogen. Vials were stored at ~20°C until ready for injection. Samples were resolved in 200 μl toluene and derivatized with 200 μl BFSTA [N,O-bis(trimethylsilyl)trifluoroacetamide] for 1 h at 70°C. Sterols were measured via gas chromatography-mass spectrometry (GC-MS), using a Shimadzu QP-2010 GC with a quadrupole mass ionizer. Peaks were identified via electron ionization (EI) fragmentation patterns. All data are relative to ergosterol content.

**Statistical analysis.** All results presented with statistical significance were analyzed with an unpaired two-tailed Student’s *t* test. A *P* of ≤0.05 was considered significant.

**RESULTS**

Inherent resistance to FLC in *A. fumigatus* is linked to an increase in erg11A transcript levels. The triazole FLC is one of the antifungals of choice for treatment of invasive fungal infections (IFIs) caused by *C. albicans* (27). However, in treatment of invasive aspergillosis (IA), FLC is ineffective due to the inherent inactivity of this triazole against *Aspergillus* spp. (31). Because FLC preferentially binds Erg11B over Erg11A, while VCZ binds both paralogs with high affinity (52), we investigated if erg11A and erg11B transcripts were differentially regulated in *A. fumigatus* during exposure to FLC or VCZ. Wild-type *A. fumigatus* was allowed to germinate in liquid culture and then dosed with a 50% MIC of FLC or VCZ for 6 and 12 h. FLC or VCZ exposure elicited increased levels of both erg11A and erg11B mRNA abundance (Fig. 1). However, the magnitude of erg11A mRNA abundance was much larger than the magnitude of erg11B mRNA abundance. VCZ treatment resulted in a 3-fold increase in erg11B mRNA, consistent with the ability of VCZ to target both erg11 paralogs in wild-type *A. fumigatus* (Fig. 1B).

![FIG 1](https://aac.asm.org/)

**Inherent resistance to FLC in *A. fumigatus* is abolished in the ΔsrbA strain and linked to erg11A transcript abundance.** In *A. fumigatus*, triazole drug resistance has primarily been observed in strains containing mutations in Erg11A that result in altered target drug affinity (2, 19, 47). However, in *C. albicans*, increases in transcript levels also contribute to triazole resistance. A similar mechanism of the erg11A/cyp51A transcript increase has recently been described in clinical samples of *A. fumigatus* (1). In the described strain, transposon integration upstream of the erg11A start codon resulted in increased expression of erg11A and drug resistance (1). Thus, increases in erg11A transcript levels may also be a clinically relevant mechanism of triazole resistance in *A. fumigatus*, although this remains to be experimentally confirmed.

We previously identified a transcription factor in *A. fumigatus*, SrbA, whose activity is critical for wild-type levels of triazole drug susceptibility (55). Yet the mechanism for the increased FLC and VCZ MICs of the ΔsrbA strain is unknown. Based on previous studies, and our initial analyses of erg11A and erg11B mRNA abundance in response to FLC and VCZ, we next examined the mRNA abundance of these transcripts in the ΔsrbA strain. A statistically significant decrease in mRNA abundance was observed for erg11A mRNA in the ΔsrbA strain compared to that in the wild type (Fig. 2) (*P = 0.03*). erg11B mRNA abundance was consistently higher in the ΔsrbA strain than erg11A but also less than in the wild type. Thus, these results suggest that a fundamental de-
crease in the erg11A transcript in the ΔsrbA strain may confer the increased susceptibility of this strain to FLC and VCZ and that SrbA may directly regulate erg11A transcript abundance.

To experimentally test this hypothesis, we asked if the erg11A and erg11B pattern of transcript induction with FLC or VCZ was altered in the ΔsrbA strain. Because the ΔsrbA strain is susceptible to FLC at a clinically relevant MIC (1.0 μg/ml [55]) and to VCZ at a MIC approximately 8-fold below the wild type, we treated mutant and wild-type germlings for 6 and 12 h postgermination at 50% of the strain MIC of each drug. When treated with FLC, erg11A mRNA abundance in the ΔsrbA strain was significantly reduced compared to that in the untreated wild-type strain (Fig. 3A). erg11B transcript levels were also reduced in the ΔsrbA strain compared to those of the wild type but not to the magnitude of erg11A. This finding is consistent with the inability of this strain to produce wild-type levels of erg11A mRNA in untreated media (Fig. 2). As with FLC, treatment with VCZ initially elicited a decrease in erg11A mRNA abundance in the ΔsrbA strain (Fig. 3B); however, prolonged exposure to VCZ, unlike FLC, was able to stimulate an increase in the erg11A transcript in the ΔsrbA strain (Fig. 3B). The subsequent increase in the erg11A transcript in response to VCZ in the ΔsrbA strain may suggest the presence of an additional transcriptional regulator of erg11A that can partially compensate for loss of SrbA function in response to VCZ. erg11B transcripts remained relatively unchanged in response to VCZ in the ΔsrbA strain. Taken together, these results suggest that SrbA may be a direct regulator of erg11A transcript levels and that decreased erg11A mRNA abundance may be responsible for the increased susceptibility of the ΔsrbA strain to FLC and VCZ.

Construction of an erg11A conditional mutant in the ΔsrbA background. In order to test our hypothesis, an erg11A conditional expression strain was constructed in the ΔsrbA background. This strain was generated by replacement of the native erg11A promoter with the nitrogen-inducible niiA promoter (23). The resulting niiA-erg11A-ΔsrbA strain genotype was verified via PCR and Southern blot analyses (Fig. 4 and data not shown). Control over erg11A mRNA abundance in the ΔsrbA background was confirmed via qRT-PCR. The induction of niiA-erg11A-ΔsrbA resulted in levels of erg11A mRNA comparable to those of the wild type. While under repressive conditions, erg11A transcript levels were similar to the ΔsrbA background (Fig. 4D).

Increased FLC and VCZ susceptibility in the ΔsrbA strain is the result of erg11A transcript insufficiency. We next characterized the effect of triazoles on the pniia-erg11A-ΔsrbA strain in inducing and repressing conditions. By using commercially available Etest strips (AB Biodisk), we determined that the FLC susceptibility phenotype of the ΔsrbA strain was completely rescued by restoration of erg11A transcript levels (Fig. 5A) (CBS 144.89, >256 μg/ml; pniia-erg11A-ΔsrbA, >256 μg/ml; ΔsrbA, 1.0 μg/ml). Importantly, FLC resistance was reversed by repression of the niiA promoter, restoring the MIC of the ΔsrbA strain prior to erg11A induction in the pniia-erg11A-ΔsrbA strain (pniia-erg11A-ΔsrbA [induced] >256 μg/ml; pniia-erg11A-ΔsrbA [repressed], 1.0 μg/ml). As the triazole VCZ binds well to both Erg11A and Erg11B, complete amelioration of the ΔsrbA strain susceptibility was not visualized in the pniia-erg11A-ΔsrbA strain (Fig. 5B) (CBS 144.89, 0.19 μg/ml; ΔsrbA, 0.008 μg/ml; pniia-erg11A-ΔsrbA, 0.064 μg/ml). However, restoration of erg11A transcript levels did increase the observed MIC, further suggesting that direct regulation of the erg11A transcript by SrbA is involved in the observed VCZ susceptibility in the ΔsrbA strain. These results likely suggest that additional unknown mechanisms influence the MIC of VCZ-treated A. fumigatus.

SrbA directly binds Erg11A upstream DNA sequence. We next asked if erg11A is directly or indirectly regulated by SrbA. An electrophoretic mobility shift assay (EMSA) was employed to de-
termine a physical interaction between an erg11A DNA motif and a recombinant form of SrbA that contained amino acids 1 to 275, which are predicted to encode the DNA binding domain. Recombinant SrbA protein was coincubated with a 118-bp biotin-labeled probe from the upstream region of the erg11A gene (Fig. 6A). This motif was chosen based on preliminary results from a chromatin immunoprecipitation-sequencing (ChIP-Seq) analysis of SrbA target genes that suggested an enrichment of SrbA binding in this region of the erg11A locus (Barker et al., unpublished data). In addition, the 118-bp probe contains a motif similar to the reported Sre1 binding motif described for S. pombe (5'-[A/T]TCA[C/A/CAT-3'] (50). A distinct shift in the probe band was observed when the protein and probe were coincubated, which was verified in the presence of a recombinant SrbA antibody through a chromatin immunoprecipitation-sequencing (ChIP-Seq) analysis of SrbA

FIG 4 Generation of erg11A inducible strain in ΔsrbA background. (A) Locus of erg11A locus in the CBS 144.89 (wild-type) strain. (B) Locus of the pniaA-erg11A construct in the ΔsrbA background strain. (C) Southern blot analysis of the wild-type and mutant strains. XbaI/XmaI genomic DNA was digested for 18 h at 37°C. Strains were separated on a 1% agarose gel, transferred, and hybridized with a 467-bp probe. (D) Induction/repression of the pniaA-erg11A-ΔsrbA strain correlates with mRNA abundance of erg11A (P = 0.008). RNA samples were extracted from each respective strain 12 h postgermination in liquid GMM plus 20 mM NO₃ (CBS 144.89, ΔsrbA, pniaA-erg11A-ΔsrbA induced [ind]) or liquid GMM plus 20 mM NH₄ (pniaA-erg11A-ΔsrbA repressed [rep]) at 37°C, 300 rpm. mRNA abundance was normalized to the housekeeping genes tefA and gadpH and are relative to CBS 144.89. Results are the means and standard deviations of three biological replicates.

A. Wild Type

B. pniaA-erg11A-ΔsrbA

C. 

D. p = 0.008

FIG 5 Restoration of erg11A transcripts restores FLC and VCZ MICs in the ΔsrbA strain. Etest strip concentrations (in micrograms per milliliter) for A. fumigatus CBS 144.89, the ΔsrbA strain, and pniaA-erg11A-ΔsrbA strain. A clear ellipse indicates susceptibility to FLC or VCZ. (A) As expected, FLC has no effect on CBS 144.89, and the ΔsrbA strain is susceptible to FLC (MIC, 1.0 μg/ml). Wild-type expression of the pniaA-erg11A-ΔsrbA strain reconstitutes FLC resistance. Likewise, repression of the pniaA-erg11A-ΔsrbA strain restores FLC susceptibility as observed in the ΔsrbA strain. (B) erg11A transcript expression partially restores the VCZ MIC in the ΔsrbA strain. CBS 144.89 MIC, 0.19 (induced or repressed); ΔsrbA strain MIC, 0.008 (induced or repressed); pniaA-erg11A-ΔsrbA strain MICs, 0.064 (induced) and 0.008 (repressed).
supershift assay (Fig. 6B). This is the first documentation of SrbA binding to any ergosterol biosynthesis gene in *A. fumigatus* and strongly suggests that SrbA plays a direct role in regulating the gene expression of *erg11A*.

Hypoxia growth defect in the ΔsrbA strain is not directly mediated by *erg11A*. Another clinically relevant phenotype of the ΔsrbA strain is its inability to grow in hypoxia, which is hypothesized to contribute to the attenuated virulence of this strain. Because the ergosterol biosynthesis pathway requires significant amounts of oxygen (26), we tested this hypothesis by examining if restoration of the *erg11A* transcript could restore the hypoxia growth phenotype of the ΔsrbA strain. The *pniIA-erg11A-ΔsrbA* conditional strain demonstrated wild-type growth rates under atmospheric oxygen (Fig. 7A and B) in both inducing and repressing conditions. Restoration of *erg11A* transcript levels, however, did not restore the ability of the ΔsrbA strain to grow in hypoxia (Fig. 7C). As the analysis of the ΔsrbA phenotype reveals that several ergosterol biosynthesis pathway genes other than *erg11A* are also transcriptionally reduced in the ΔsrbA strain (55), blockages further downstream of *erg11* likely contribute to this growth defect (7a).

To verify that the lack of growth in the ΔsrbA strain is not solely due to *erg11A*, we examined Δ*erg11A* and *pniIA-erg11A-Δerg11B* conditional strains for growth under hypoxic conditions. Both the Δ*erg11A* and *pniIA-erg11A-Δerg11B* conditional strains grew in hypoxia at rates comparable to those in normoxia (Fig. 8), independent of nitrogen source. As expected, the *pniIA-erg11A-Δerg11B* strain was lethal under repressing conditions (23). We thus conclude that the hypoxia growth defect in the ΔsrbA strain is not directly due to *erg11A* deficiency and therefore may be due to affected ergosterol biosynthesis genes further downstream of *erg11A*.

Total ergosterol content is not altered with *erg11A* transcript restoration in the ΔsrbA strain. To test the hypothesis that restoration of the *erg11A* transcript does not rescue hypoxia growth due to continued perturbations in the ergosterol pathway, we compared the ergosterol content of the ΔsrbA strain with that of the wild type (Fig. 9). Total ergosterol was measured via high-resolution nuclear magnetic resonance (NMR) spectroscopy. The results showed that the total ergosterol content is not significantly different between the ΔsrbA and wild-type strains (55).

**FIG 6** SrbA directly binds *erg11A* promoter region sequence in vitro. Electrophoretic mobility shift assays were conducted utilizing a LightShift chemiluminescent EMSA kit from Thermo Scientific. A total of 40 fmol of a 118-bp biotin-labeled probe from *erg11A* upstream region and 1 μg purified SrbA protein were coincubated at room temperature for 20 min. Recombinant polyclonal SrbA antibody was used for the supershift assay. Lane 1, no protein control; lane 2, EMSA reaction; lane 3, supershift assay.

**FIG 7** Restoration of *erg11A* does not affect hyphal growth in normoxia or restore ΔsrbA-related hypoxic growth defect. A total of 10⁵ conidia was spotted on solid media (GMM plus 20 mM NO₃ [induction] or 20 mM NH₄ [repression]) under normoxic (N) or hypoxic (H) conditions. (A) Comparison of colony growth diameter on nitrate (NO₃) in both hypoxia (H) and normoxia (N). (B) Comparison of colony growth diameter on ammonium (NH₄) in both hypoxia (H) and normoxia (N). Values represent the means and standard deviations of 3 biological replicates. (C) Radial growth at 120 h.
performance liquid chromatography (HPLC) and all concentrations were based on peak intensity comparisons with a standard curve of commercially available ergosterol (Acros Organics). Twelve hours after germination, the \textit{H9004 srbA} strain contained approximately 24.8\% of the ergosterol content found in the wild type (5.71 \pm 1.02 \text{ g/mg} versus 2.36 \pm 0.79 \text{ g/mg}, respectively). Strikingly, induction of \textit{erg11A} in the \textit{H9004 srbA} background had no effect on total ergosterol production (1.85 \pm 0.89 \text{ g/mg} versus 2.36 \pm 0.79 \text{ g/mg}, respectively). This result is consistent with our hypothesis that SrbA regulates multiple genes in the ergosterol biosynthesis pathway.

To further support this hypothesis, we analyzed the production of sterol intermediates in our strains. Previous qualitative sterol profiles of the \textit{H9004 srbA} strain demonstrated an increase in 4-methyl sterols compared to those of wild-type \textit{A. fumigatus} (55). Buildup of these 4-methyl sterols indicates a significant blockage at the Erg25 (C4-demethylase) step in the pathway. In particular, transcriptional analysis of the \textit{H9004 srbA} strain also indicates that the \textit{erg25A} transcript is significantly repressed (55), suggesting that SrbA is also involved in regulation of this gene. Intriguingly, both steps at Erg11 and Erg25 require oxygen and iron as cofactors (9, 7a, 28, 50). Because restoration of the \textit{erg11A} transcript in the \textit{SrbA} strain does not increase total ergosterol levels (Fig. 9), we hypothesized that there was a corresponding increase in 4-methyl sterol intermediates in this strain. This hypothesis was based on the premise that SrbA regulates both \textit{erg11A} and \textit{erg25} and that restoration of \textit{erg11A} enzymatic activity would increase levels of intermediates in the first half of the pathway. Due to the continued blockage at Erg25, an increase in intermediates corresponding to this step would be observed, which would correlate with the lack of increase in total ergosterol. We thus examined the sterol intermediate profiles of both the \textit{SrbA} and \textit{piiiA-erg11A-SrbA} strains via GC-MS and compared their profiles to those of wild-type CBS 144.89. As previously observed, an increase in 4-methyl sterols was detected in the \textit{SrbA} strain (Fig. 10). Consistent with an unalleviated blockage at Erg25 in the \textit{piiiA-erg11A-SrbA} strain, three peaks corresponding with buildups in 4-methyl sterols were increased in the \textit{piiiA-erg11A-SrbA} strain under inducing conditions (Fig. 10 [4,14,24-trimethyl cholesta-8,24(28)-dien-3\textbeta\text{-ol}, \~75\% based on peak identification; 4,24-dimethyl cholesta-8(28)-dien-3\textbeta\text{-ol} and 4,4,24-trimethyl cholesta-8(28)-dien-3\textbeta\text{-ol}, \~85\% identity based on peak identification]). Taken together, these results suggest that the \textit{SrbA} strain also contains a blockage downstream of Erg11, most likely in the C4-sterol oxidase enzyme, Erg25. Experiments are under way to determine if alleviation of this blockage can rescue the hypoxia growth and virulence phenotypes of the \textit{SrbA} strain.

**DISCUSSION**

Our study highlights the importance of SrbA in the regulation of ergosterol biosynthesis in \textit{A. fumigatus} and provides further mechanistic insight into the inherent FLC resistance and triazole susceptibility of this important human pathogenic fungus. We began this work with the hypothesis that the reduction of the \textit{erg11A} transcript in the \textit{SrbA} strain was the likely mechanism by which the \textit{SrbA} strain is susceptible to the triazole FLC and has increased susceptibility to VCZ (55). By constructing an inducible \textit{erg11A} strain (\textit{piiiA-erg11A-SrbA}), we have demonstrated that

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**FIG 8** Genetic deletion of \textit{erg11A} (\textit{Δerg11A}) or \textit{erg11B} (\textit{piiiA-erg11A-Δerg11B}) does not affect hyphal growth in normoxia or hypoxia. A total of 10^5 conidia was spotted on solid media (GMM plus 20 mM NO\textsubscript{3} [induction] or 20 mM NH\textsubscript{4} [repression]) under normoxic or hypoxic conditions. (A) Radial growth at 96 h. (B) Comparison of colony growth diameter on nitrate (NO\textsubscript{3}) in both hypoxia and normoxia. (C) Comparison of colony growth diameter on ammonium (NH\textsubscript{4}) in both hypoxia and normoxia. Values represent the means and standard deviations of 3 biological replicates.

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**TABLE A**

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<thead>
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<tr>
<td>CBS 144.89</td>
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<tr>
<td>\textit{Δerg11A} (NO\textsubscript{3})</td>
<td>2.36 ± 0.79</td>
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<tr>
<td>\textit{erg11A-ΔsrbA} (NO\textsubscript{3})</td>
<td>1.85 ± 0.89</td>
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<tr>
<td>\textit{erg11A-Δerg11B (NO\textsubscript{3})}</td>
<td>2.36 ± 0.79</td>
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<tr>
<td>\textit{erg11A-Δerg11B (NH\textsubscript{4})}</td>
<td>2.36 ± 0.79</td>
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**TABLE B**

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<td>\textit{erg11A-Δerg11B (NH\textsubscript{4})}</td>
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**TABLE C**

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FIG 9  Total ergosterol is unchanged by restoration of erg11A transcript levels in the ΔsrbA strain. Total ergosterol from A. fumigatus strains was extracted 12 h postgermination in liquid GMM with 20 mM NO₃ (niiA-erg11A-ΔsrbA ind, ΔsrbA, and CBS 144.89) or liquid GMM with 20 mM NH₄ (niiA-erg11A-ΔsrbA rep). Mycelia were harvested via vacuum filtration and then dried and weighed. Total ergosterol was measured using a Shimadzu CLASS-VP HPLC and detected via an SPD-M10A VP PDA at 280 nm on a μBondapack C₁₈ column (300 mm by 3.9 mm, 10 μm). *, P < 0.001. Values represent the means and standard deviations of 3 to 5 biological replicates and three technical replicates.

restoration of wild-type levels of the erg11A transcript restores the FLC resistance observed in A. fumigatus and also restores wild-type levels of VCY2 susceptibility (Fig. 5). We therefore have confirmed that this regulation of erg11A by SrbA is likely direct, as is demonstrated by direct binding of recombinant SrbA to the promoter region of erg11A. Furthermore, this region contains a sequence similar to the SREBP DNA binding motif reported for S. pombe, though additional analyses of the actual SrBα DNA binding motif remain to be performed (Fig. 6).

Thus, the apparent regulation of erg11 by SrbA may suggest an additional target for therapeutic control over this important pathway in human pathogenic fungi. Though clinical resistance to triazoles via a defined transcriptional mechanism has not yet been reported for A. fumigatus, our research may suggest a role for SrbA in the development of triazole resistance, as alterations in this transcription factor or its DNA binding affinity could transcriptionally initiate changes that alter target abundance and lead to triazole resistance. This possibility remains to be experimentally or clinically confirmed.

Precedence for the idea that alterations in a transcription factor or transcription factor DNA binding sites can affect drug resistance is found in the pathogenic yeast C. albicans, in tumorigenesis, and in the malarial parasite Plasmodium vivax (15–17, 35, 39, 44, 58). For example, mutations in C. albicans transcription factors that regulate drug efflux pumps are more susceptible to antifungal drugs (16, 39, 44, 58). Additionally, a mutation in the regulator of erg11 in C. albicans, Upc2, renders the mutant more susceptible to triazole antifungals. In concordance with the role of Upc2 in regulation of erg11, a heterozygous gene replacement mutant of Upc2 (UPC2/upc2) and a homozygous gene replacement mutant (upc2/upc2) in a strain with two Upc2 alleles both exhibited increased susceptibility to the triazole antifungals FLC and itraconazole (48). upc2 gene deletion mutants have also been constructed in a strain containing four upc2 alleles, which is thought to occur from chromosomal polysomy, and is observed occasionally in strains cultivated from patients (54). In these strains, genetic removal of two or more of the upc2 alleles conferred a significant susceptibility to theazole ketoconazole or the triazole FLC (30). The upc2 deletion mutant from S. MacPherson et al. (30) was also unable to grow in hypoxia. Overall, both of the described C. albicans upc2 deletion mutants share strikingly similar phenotypes to that of the ΔsrbA strain of A. fumigatus and provide further support for our idea that alterations in the transcription factor SrbA may be worth monitoring in the context of triazole-resistant clinical strains of A. fumigatus with unknown resistance mechanisms.

Although Upc2 is not a homologue of SREBPs, these two classes of transcription factors have analogous functions, similar localization and activation patterns, and are proposed to be an example of convergent evolution in the fungal kingdom (7, 21, 32, 46, 54). The inactive protein of both classes is tethered to a cytoplasmic membrane, and both are released via proteolytic cleavage to function as transcription factors (21, 46, 54). Also, genetic deletion of either Upc2 or SREBPs results in decreased accumulation of ergosterol, increased susceptibility to antifungal drugs, and an inability to induce expression of erg11 under antifungal drug pressure (21, 30, 46, 54). Unlike SREBPs, Upc2 seems to have no effect on the basal regulation of erg11, as levels of erg11 are unchanged in the upc2Δ strain without the addition of antifungal drugs (21, 46).

With regard to antitumor drugs and the malarial parasite P. vivax, these pathosystems illustrate further examples of drug resistance mediated by transcription factor mutations (15–17, 35, 39, 44, 58). Ex vivo whole-genome sequencing and microarray analysis of a chloroquine-resistant P. vivax isolate identified six transcription factors with aberrantly high levels. These six transcription factors all contained high levels of nonsynonymous mutations and belonged to the AP class of transcription factors. These nonsynonymous mutations are thought to cause increased expression of downstream targets, resulting in drug resistance at a transcription regulation level. These results have raised the question if modification of transcription factors may be more common in antimalarial drug resistance than previously acknowledged. The authors argue that understanding this understudied resistance...
mechanism will assist in the treatment of patients with malaria and extend the usefulness of the drug chloroquine (15). Likewise, we argue that further study of the role of fungal transcription factors and antifungal drug resistance in *A. fumigatus* is likely needed in a similar context to provide additional insights into new antifungal drug design. A recent study from Albarrag et al. identified a transposon-mediated insertion into the promoter region of *erg11A/cyp51A* in a triazole-resistant clinical isolate of *A. fumigatus*. This strain has higher levels of the *erg11A/cyp51A* transcript, but its direct association with the transposon insertion is currently unknown and awaits further investigation (1).

The partial restoration of the Vez CMC in the *pniA-erg11A*ΔsrbA strain is another potentially interesting finding. This result suggests that additional SREBP-dependent or off-target effects exist in the *pniA-erg11A*ΔsrbA strain. The persistent depletion of total ergosterol may be one mechanism whereby the *pniA-erg11A*ΔsrbA strain regains only a portion of the wild-type susceptibility to VezC. The stability of V-type ATPases, critical for maintaining vacuolar ion homeostasis, is directly related to the structural integrity of the cellular membrane and is affected by depletions of ergosterol concentration in *C. albicans* (57). Therefore, a persistent decrease of total ergosterol content in the *pniA-erg11A*ΔsrbA strain, in conjunction with the dual target capabilities of Vcz, could lend itself to the partial restoration of VezC susceptibility observed in the *pniA-erg11A*ΔsrbA strain. As FLC only strongly binds *erg11B*, restoration of *erg11A* mRNA abundance appears to be sufficient for resistance to this triazole, and off-target effects are either not observed or inefficient in the *pniA-erg11A*ΔsrbA strain.

An additional important phenotype of the SrbA null mutant investigated here is its inability to grow in hypoxia and to cause lethal disease in murine models of invasive aspergillosis. Whether the attenuated virulence of fungal SREBP strains is due to their inability to adapt to *in vivo* hypoxia is still an unanswered question and is also relevant from the perspective of antifungal drug development. Thus, we explored whether restoration of the *erg11A* transcript could also restore the hypoxia growth phenotype of the ΔsrbA strain. Although restoration of the *erg11A* transcript did not restore the ability of the ΔsrbA strain to grow in hypoxia, both Δ*erg11A* and *pniA-erg11A*ΔsrbA strains also grow at wild-type rates in hypoxia (Fig. 8). Thus, blockages at Erg11 appear to not affect the ability of *A. fumigatus* to grow in hypoxia. We speculate that additional blockages downstream of Erg11 may play a more critical role. Further evidence for this hypothesis is demonstrated by the decrease in total ergosterol content (Fig. 9) and abundance of 4-methyl sterols (Fig. 10) in the *pniA-erg11A*ΔsrbA strain. The intermediates identified in the sterol profile indicate that the primary blockage in both ΔsrbA and *pniA-erg11A*ΔsrbA strains occurs around the C4-demethylation step, which is initiated by the C4-methyl sterol oxidase (*erg25*).

*erg25* expression is induced in response to itraconazole treatment in the human pathogenic fungus *C. albicans* (13). This transcript is also increased 3-fold in *C. albicans erg11* mutants (42). In *Saccharomyces cerevisiae*, mutations in *erg25* are generally lethal but can be rescued by sterol supplementation. Thus, the *erg25* gene replacement mutant is a sterol auxotroph in *S. cerevisiae* (20). Interestingly, the phenotypes of this mutant can be rescued by the addition of triazole antifungals, suggesting that the accumulation of 4,4,24-trimethyl cholesta-8,24(28)-dien-3β-ol is toxic to the yeast and that the corresponding reduction in *erg11* is required for membrane integrity (20). Although this sterol intermediate is observed at high levels in both ΔsrbA and *pniA-erg11A*ΔsrbA strains, it does not seem to have a toxic side effect on *A. fumigatus*, as these mutants grow at wild-type levels (Fig. 7). Potential toxic effects of this sterol intermediate may be negated, however, by the presence of adequate ergosterol, theoretically generated by ergosterol biosynthesis genes not in the regulon of SrbA, possibly such as *erg11B*. Experiments to determine the role of blockages at the C4-methyl sterol oxidase step of ergosterol biosynthesis in the ΔsrbA strain are ongoing.

In conclusion, our study further emphasizes the important link between fungal SREBPs, ergosterol biosynthesis, and triazole drug interactions. We show that transcriptional regulation of the triazole target *erg11* in *A. fumigatus* by SrbA is an important mechanism that explains the FLC susceptibility and increased VezC susceptibility of this strain. However, regulation of the *erg11A* transcript by SrbA does not directly appear to affect the ability of *A. fumigatus* to grow in hypoxia. Further work is needed to definitively determine what parts of the ergosterol biosynthesis pathway are directly regulated by SrbA and whether this regulation is the major mechanism behind the inability of this strain to grow in hypoxia and cause lethal disease. We also propose that examination of mutations in the DNA binding domain of SrbA or mutations in its DNA binding sequence motif may also be important future lines of inquiry in triazole-resistant isolates that do not contain one of the canonical resistance mechanisms.

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SREBP Mediates A. fumigatus Triazole Susceptibility


