The SET-domain protein CgSet4 negatively regulates antifungal drug resistance via the ergosterol biosynthesis transcriptional regulator CgUpc2a

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Invasive fungal infections, which pose a serious threat to human health, are increasingly associated with a high mortality rate and elevated health care costs, owing to rising resistance to current antifungals and emergence of multidrug-resistant fungal species. Candida glabrata is the second to fourth common cause of Candida bloodstream infections. Its high propensity to acquire resistance toward two mainstream drugs, azoles (inhibit ergosterol biosynthesis) and echinocandins (target cell wall), in clinical settings, and its inherent low azole susceptibility render antifungal therapy unsuccessful in many cases. Here, we demonstrate a pivotal role for the SET [suppressor of variegation 3 to 9 (Su(var)3–9), enhancer of zeste (E(z))], and trithorax (Trx)] domain–containing protein, CgSet4, in azole and echinocandin resistance via negative regulation of multidrug transporter–encoding and ergosterol biosynthesis (ERG) genes through the master transcriptional factors CgPdr1 and CgUpc2A, respectively. RNA-Seq analysis revealed that C. glabrata responds to caspofungin (CSP; echinocandin antifungal) stress by downregulation and upregulation of ERG and cell wall organization genes, respectively. Although CgSet4 acts as a repressor of the ergosterol biosynthesis pathway via CgUPC2A transcriptional downregulation, the CSP-induced ERG gene repression is not dependent on CgSet4, as CgSet4 showed diminished abundance on the CgUPC2A promoter in CSP-treated cells. Furthermore, we show a role for the last three enzymes of the ergosterol biosynthesis pathway, CgErg3, CgErg5, and CgErg4, in antifungal susceptibility and virulence in C. glabrata. Altogether, our results unveil the link between ergosterol biosynthesis and echinocandin resistance and have implications for combination antifungal therapy.

Fungal infections, whose incidence has been on the rise over last 2 decades, are associated with a mortality rate of as high as 95% (1–5). Infections caused by the Candida species are the most prevalent cause of hospital-acquired bloodstream fungal infections, with Candida albicans being the predominant species (1, 2, 4, 5). Candida glabrata is emerging as the first to third most prevalent non-albicans Candida species, after Candida tropicalis and Candida parapsilosis (1, 2, 4–6).

C. glabrata is an asexual haploid budding yeast, which belongs to the Nakaseomyces clade (7, 8). It shares a common ancestor with the non-pathogenic yeast Saccharomyces cerevisiae and is distinct in its virulence traits from other prevalent pathogenic species of Candida (7, 8). C. glabrata lacks secreted proteolytic activity, does not form true hyphae, and displays elevated levels of stress resistance (7, 8). Based on its position in the phylogenetic tree and close genetic relatedness with S. cerevisiae, pathogenesis mechanisms in C. glabrata are postulated to have arisen independently of other Candida pathogens (7, 8). The ability to survive and replicate in human macrophages, suppress host innate immune response, form biofilms, adhere to host tissue, metabolic flexibility and high resistance to diverse stresses including antifungals, assist C. glabrata establish successful mucosal and invasive infections (1, 7, 8).

The incidence of C. glabrata infections has been on rise since last 2 decades, and alarming, this surge is also associated with an increased prevalence of co-resistance to two mainstream antifungal agents, azoles and echinocandins, thereby, hampering the success of antifungal therapy (3–5, 9–12). Polyene, azole, and echinocandin drugs are largely being used in hospitals worldwide to treat bloodstream fungal infections (11–13). The target of azole antifungals, which are fungistatic, is an enzyme of the ergosterol biosynthesis pathway, lanosterol 14-alpha-demethylase enzyme, that is encoded by the ERG11 gene (13, 14). Azole drug–induced growth inhibition is attributed to ergosterol depletion from the plasma membrane and accumulation of the toxic sterol intermediates (13, 14). Contrary to azoles, the echinocandin antifungals target the β-glucan synthase enzyme, which is encoded by the family of FKS genes, and is involved in the synthesis of the cell wall structural polymer 1,3-β-D-glucan (11–13, 15). The polyene class of antifungals bind to ergosterol and disrupt plasma membrane integrity as well as extract ergosterol from the cell membrane (12, 13).

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C. glabrata is inherently less susceptible to azole drugs, and C. glabrata infections are increasingly being associated with azole and/or echinocandin resistance (4, 5, 9–12). The elevated expression of ATP-binding cassette class of multidrug transporters is the primary mode of azole resistance in C. glabrata in hospitals worldwide (11, 12, 14). Genes coding for multidrug transporters are under the regulation of a Zn$_2$Cys$_6$ binuclear zinc cluster domain—containing transcription factor, which is encoded by the CgPDR1 gene (14, 16). A large number of single amino acid substitution mutations in CgPDR1 have been reported in azole-resistant clinical isolates of C. glabrata (14, 17, 18). CgCdr1 and CgCdr2 represent two major multidrug transporters in C. glabrata, whose elevated expression is associated with increased azole efflux, and the resultant decreased intracellular accumulation of azole drugs (14, 19, 20).

Another key regulator of azole antifungal response is the Zn$_2$Cys$_6$ transcription factor CgUsc2A (Cagl0c01199p), which is required for basal and induced expression of the ergosterol biosynthesis (ERG) genes, with several ERG genes showing significant upregulation upon azole exposure (21–23). Intriguingly, CgUsc2A has recently been shown to bind to CgPDR1 and CgCDR1 promoter sequences, thereby linking ergosterol biosynthesis with the CgPdr1-mediated transcriptional regulatory network that controls expression of multidrug resistance (MDR) genes (24).

C. glabrata possesses three CgFKS genes, and point mutations in the hot spot regions of CgFKS1 and CgFKS2 genes largely account for echinocandin resistance in clinical isolates of C. glabrata (15, 25). These hot spot regions represent echinocandin-binding regions, with mutations decreasing the binding affinity of echinocandins for the β-1,3-d-glucan synthase enzyme (12, 15). Besides elevated CgFKS gene transcription, the echinocandin exposure also leads to increased expression of the chitin synthase genes, thereby resulting in compensatory activation of chitin synthesis in the cell wall (15, 26). Mutations in the CgMSH2 gene, that codes for a component of the DNA mismatch repair pathway, have been associated with MDR in some isolates of C. glabrata (11, 15).

We have recently reported that disruption of CgSET2 (encodes a histone H3 lysine 36 methyltransferase), CgSET4 (contains evolutionarily conserved SET {suppressor of variegation 3 to 9 [Su(var)3–9], enhancer of zeste [E(z)], and triatorch (Trx)} domain), and CgRPH1 (encodes a putative histone lysine demethylase) genes resulted in azole resistance, resistance, and sensitivity, respectively (27, 28). Notably, these C. glabrata proteins have orthologs in S. cerevisiae, Set2, Set4, and Rph1, which have been studied for their role in chromatin homeostasis (29–32). Rph1, a HmJc domain–containing histone lysine demethylase, and Set2, a methyltransferase, have been implicated in demethylation and methylation of the lysine 36 residue in histone H3, respectively, in S. cerevisiae (29, 32). Set4 in S. cerevisiae has been shown to be a stress-responsive chromatin-associated protein that regulates gene expression under oxidative stress conditions, acts as a repressor of ergosterol biosynthesis, and aids in the maintenance of a repressive environment at subtelomeres (31, 33, 34). However, the epigenetic regulation of MDR genes is largely unstudied in C. glabrata.

Here, we report that of six SET domain proteins (CgSet1–CgSet6) in C. glabrata, CgSet4 uniquely acts as a repressor of CgPdr1-dependent MDR and CgUsc2a-dependent ergosterol biosynthesis pathways. Besides showing that CgSET4 deletion results in decreased susceptibility to fluconazole (FLC) and caspofungin (CSP) drugs, we also report that ergosterol biosynthesis is downregulated in response to CSP stress. We further show that CgSet4-dependent negative regulation of CgPDR1 and CgERG genes is mediated through CgUpc2a. Finally, our animal infection studies reveal a role for the master transcriptional factor CgUpc2a, and two enzymes (CgEr3 and CgEr4) of the ergosterol biosynthesis pathway, as well as, for all CgSet proteins but for CgSet6, in survival of C. glabrata in the mouse systemic candidiasis model. Besides uncovering a link between ergosterol biosynthesis and echinocandin resistance, our findings yield key insights into the intertwined transcriptional networks that regulate cellular response to two seemingly distinct stresses, cell wall impairment, and ergosterol synthesis inhibition.

Results

The SET domain–containing protein CgSet4 negatively regulatesazole and CSP resistance

We have previously shown a role for putative histone chaperones CgFpr3 and CgFpr4, histone demethylase CgRph1, and histone H3K36-specific methyltransferase CgSet2 in regulating CgPDR1-dependent expression of multidrug transporter–encoding genes, and/or resistance to azole antifungals (28). Extending our results further, we, here, have examined the contribution of C. glabrata proteins, which possess the evolutionarily conserved SET domain, to resistance toward azole and echinocandin drugs. The SET domain consists of about 130 conserved amino acids (35). Several SET domain–containing proteins are known to methylate both histone and nonhistone proteins, with ε-amino group of the lysine residue in histones being able to undergo mono-methylation, dimethylation, and trimethylation (35–37). Through in silico analysis, we first identified six genes, CgSET1–SET6, in the C. glabrata genome, which code for proteins containing the SET domain. The key features of these six SET domain–containing proteins CgSet1–CgSet6 and functions of their S. cerevisiae orthologs are listed in Table S1. The amino acid sequence alignment of the SET domain, and the predicted domain organization of CgSet1–Set6 proteins are shown in Fig. S1, A and B, respectively. Notably, CgSet1 and CgSet2 enzymes have recently been shown to be required for monomethylation, dimethylation, and trimethylation of lysine-4, and trimethylation of lysine-36 residues, in histone H3, respectively (28, 38), while this work was underway.

Next, we generated and characterized deletion strains for CgSet1, and CgSet3–6 genes, as we had recently shown that the loss of CgSet2 gene resulted in decreased sensitivity to the azole drug FLC (28). In consistence with the recent studies (28, 38), phenotypic analysis showed increased sensitivity and a
survival in mice (28), we next assessed the survival of the SET domain–containing proteins in C. glabrata. We found that unlike ScSet3, ScSet4, and ScSet5 proteins in antifungal toler-ance, these data collectively implicate CgSet4 in resistance to FLC and CSP (Fig. 2A and B), suggesting that CgSet4 negatively regulates resistance toward FLC and CSP drugs in C. glabrata.

CgSet4 is an uncharacterized 350 amino acid protein that showed 40% identity with its S. cerevisiae ortholog and contains a 132 amino acid (146–277 amino acids) SET domain (Fig. S1B). In S. cerevisiae, Set4 has a paralog, Set3; however, neither Set3 nor Set4 has been reported to possess lysine methyltransferase activity (31, 39). Similarly, we found normal H3K4me3 and H3K36me3 levels in the Cgset4A mutant (Fig. 1E). The amino acid sequence similarity and the lack of histone H3K4 and H3K36 methyltransferase activity in both CgSet4 and ScSet4 proteins prompted us to examine if ScSet4 expression could reverse antifungal resistance in the Cgset4A mutant. For this, we ectopically expressed the S. cerevisiae SET4 from the endogenous CgSET4 promoter in the Cgset4A mutant and compared growth profiles of the Cgset4A mutant expressing either ScSET4 or CgSET4, in the presence of antifungals. We found that unlike CgSet4, ectopic expression of the S. cerevisiae SET4 could not rescue FLC and CSP resistance phenotype of the Cgset4A mutant (Fig. 2, A and B), indicating functional differences between CgSet4 and ScSet4 proteins in modulation of antifungal resistance. Of note, while the SET4 gene loss led to azole resistance, it had no effect on CSP susceptibility in S. cerevisiae (33).

To examine the role of CgSet4 in other clinical strains of C. glabrata, we deleted CgSET4 gene in the reference strain CBS138 and two Indian clinical isolates YRK2289 and YRK2291 (27). The CgSET4 loss resulted in decreased susceptibility to FLC (Fig. S2A) and CSP (Fig. S2B) in all three
Figure 1. The loss of SET domain–containing protein, CgSet4, leads to azole and echinocandin resistance. A, serial dilution spotting analysis illustrating fluconazole (FLC) susceptibility of indicated *Candida glabrata* strains. Overnight-grown *C. glabrata* cultures were 10-fold serially diluted, and 3 μl of each dilution was spotted on the CAA medium lacking (CAA) or containing 16 μg/ml FLC (FLC-16), 32 μg/ml FLC (FLC-32), or 64 μg/ml FLC (FLC-64). Images were captured after 2 days of growth at 30 °C. "wt" denotes the wild-type (wt) strain. B, serial dilution cell spotting analysis illustrating oxidative stress and DNA damage stress susceptibility of indicated *C. glabrata* strains. Hydrogen peroxide (H2O2) and methyl methane sulfonate (MMS) were used at a concentration of 35 mM and 0.04%, respectively, in YPD medium. C, serial dilution cell spotting analysis illustrating cell wall stress susceptibility of indicated *C. glabrata* strains. Both calcofluor white (CFW) and Congo Red were used at a concentration of 2 mg/ml in CAA medium. D, liquid medium–based growth analysis illustrating caspofungin (CSP) susceptibility of indicated *C. glabrata* strains. *C. glabrata* strains were cultured at 30 °C in CAA medium lacking (CAA) or containing 75 ng/ml CSP (CSP-75) or 150 ng/ml CSP (CSP-150) for 16 h. After incubation, cultures were diluted in PBS, and 3 μl of 5-, 25-, 125-, and 625-fold diluted cultures were spotted on CAA medium, and growth was recorded after 1 day of growth at 30 °C. E, representative immunoblots showing trimethylation on 4th and 36th lysine residues in histone H3 in indicated *C. glabrata* strains. Log-phase wt and CgsetΔ cells were collected, washed with PBS,
strain backgrounds. Furthermore, CgSET4 overexpression led to FLC (Fig. S2A) and CSP (Fig. S2B) sensitivity in CBS138 and Indian clinical strains, albeit to different extent. These results suggest that CgSet4-dependent control of antifungal resistance is common among clinical strains of C. glabrata.

Next, to delineate the role of CgSet4 in antifungal resistance, we decided to first check its cellular localization. For this, we generated CgSet4-GFP fusion protein and found it to be functional, as it complemented FLC resistance phenotype of the Cgset4Δ mutant (Fig. S3A). Next, the confocal imaging analysis revealed that CgSet4 is primarily localized to the nucleus under all (regular, FLC-treated, and CSP-treated) conditions (Fig. 2C), indicating a predominant nuclear role for CgSet4 in C. glabrata. Furthermore, CgSet4-GFP was found to be highly enriched in the insoluble chromatin fraction, whereas being totally absent in the soluble cytosolic fraction (Fig. S3B), indicating that CgSet4 is a chromatin-associated protein.

Since CgSET4 gene loss was associated with drug resistance, we next checked the effect of antifungal treatment on CgSET4 transcript and protein levels. For this, we compared CgSET4 transcript and protein levels, via quantitative RT–PCR (qRT–PCR) and Western analysis, respectively, between untreated and FLC- or CSP-treated wt cells (Fig. 2D). We found that CgSET4 transcript (Fig. 2D) and CgSet4-GFP (~58 kDa band) protein (Fig. 2E) levels were about two- to five-fold lower in FLC- and CSP-treated wt cells, compared with untreated wt cells, indicating that C. glabrata responds to azole and echinocandin exposure by downregulating CgSET4 expression. Of note, this antifungal-induced CgSET4 repression is in accordance with the FLC and CSP resistance observed in the Cgset4Δ mutant (Fig. 1, A and D). Collectively, our data suggest that CgSet4 is a nuclear protein that may be a pivotal component of the azole and echinocandin antifungal resistance regulatory system in C. glabrata.

CgSet4 negatively regulates the CgPDR1 regulon

Since the azole resistance in C. glabrata is primarily associated with overexpression of the multidrug transporter–encoding genes CgCDR1 and CgCDR2, owing to increased protein or activity levels of CgPdr1 (14), we next checked the transcript levels of CgPDR1, CgCDR1, and CgCDR2 genes in Cgset4Δ mutant. We found that 1.8-, 2.7-, and 2.7-fold increased expression of CgPDR1, CgCDR1, and CgCDR2 genes, respectively, in the Cgset4Δ mutant (Fig. 3A). Furthermore, while FLC exposure resulted in elevated CgPDR1, CgCDR1, and CgCDR2 transcript levels in wt cells, no such increase was observed in FLC-treated Cgset4Δ cells, compared with untreated Cgset4Δ cells (Fig. 3A). These data suggest that FLC resistance in the Cgset4Δ mutant could be due to high basal level expression of CgPDR1 regulon genes.

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To verify this notion, we deleted CgCDR1 gene from the genome of both wt and Cgset4Δ strains. We found that while CgCDR1 gene loss rendered wt cells highly susceptible to FLC, CgCDR1 gene loss in the Cgset4Δ mutant reversed the azole resistance phenotype of the mutant, as the double mutant Cgset4Δcdr1Δ exhibited wt-like sensitivity to FLC (Fig. 3B). Importantly, expression of CgSET4 and CgCDR1 genes in the Cgset4Δcdr1Δ mutant led to elevated and diminished FLC susceptibility, similar to Cgcdr1Δ and Cgset4Δ mutant, respectively (Fig. 3B). Of note, the wt-like and not the Cgcdr1Δ-like FLC susceptibility of the double mutant Cgset4Δcdr1Δ raises the possibility that genes other than CgCDR1 may also contribute to FLC resistance in the Cgset4Δ mutant. Despite multiple attempts, we could not generate the double deletion strain Cgset4Δpdr1Δ lacking both CgSET4 and CgPDR1 genes, the reason underlying this is yet to be determined. Collectively, besides showing that FLC resistance in the Cgset4Δ mutant is predominantly dependent upon CgCdr1, these results implicate CgSet4 in the transcriptional repression of CgPDR1 in C. glabrata.

Cell wall composition is not altered in the Cgset4Δ mutant

After elucidating the molecular basis of azole resistance, we next focused on the echinocandin resistance in the Cgset4Δ mutant. CSP inhibits synthesis of the cell wall component, β-1,3-d-glucan, and a compensatory increase in cell wall chitin levels has been reported upon CSP exposure (15, 26, 41). Therefore, we next checked if CSP resistance in Cgset4Δ mutant could be due to an altered cell wall composition in the mutant. For this, we determined levels of all three components of the cell wall, viz., β-glucan, mannan, and chitin, via aniline blue, concanavalin A, and calcofluor white–based cell staining assays, respectively. Fluorescence-activated cell sorting analysis showed similar mannan and chitin content in cell walls of the wt and Cgset4Δ strains, and a very mild increase in β-glucan levels in the mutant cell wall, as compared with the wt cell wall (Fig. S4), thereby ruling out any significant contribution of the cell wall components to CSP resistance in the Cgset4Δ mutant.

Ergosterol biosynthesis gene expression is downregulated in response to CSP exposure

CgSet4 was found to be associated with chromatin (Fig. S3B). Therefore, to uncover the molecular basis of CSP resistance in the Cgset4Δ mutant, we next profiled the global transcriptomes, using RNA-Seq approach, of logarithmic phase wt and Cgset4Δ cells in the presence and absence of CSP. For this, C. glabrata cells were treated with CSP at a sublethal concentration (0.25 μg/ml) for 1 h, and both wt and Cgset4Δ cells were found to retain viability during this treatment period (Fig. S5).

and lysed with glass beads. Whole-cell lysates (50 μg protein) were resolved on 15% SDS-PAGE and probed with anti-H3, anti-H3K4me3, anti-H3K36me3, and anti-GAPDH antibodies. Bands representing histone H3 and CgGapdh proteins corresponded to about 17 and 36 kDa, respectively. CgGapdh was used as a loading control. F. organ fungal load in 6- to 8-week-old female BALB/c mice was determined 7 days postintravenous infection with indicated C. glabrata strains (4 × 10⁶ cells). Diamonds and bars denote CFUs recovered from target organs of the individual mouse, and the CFU geometric mean (n = 7–10), respectively, for each organ. *p < 0.05; **p < 0.01; ***p < 0.001; Mann–Whitney test. CAA, casamino acid; CFU, colony-forming unit; SET, (suppressor of variegation 3 to 9 [Su(var)3–9], enhancer of zeste [Elz]), and trithorax (Trx); YPD, yeast extract–peptone–dextrose.
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Figure 2. Antifungal exposure results in diminished transcript and protein levels of the CgSET4 gene. A, serial dilution spotting analysis illustrating fluconazole (FLC) susceptibility of indicated Candida glabrata strains. FLC was used at a concentration of 16 μg/ml (FLC-16), 32 μg/ml (FLC-32), and 64 μg/ml (FLC-64) in CAA medium. "V" CgSET4, and ScSET4 denote empty vector, C. glabrata SET4, and Saccharomyces cerevisiae SET4 gene, respectively. B, liquid medium–based growth analysis illustrating caspofungin (CSP) susceptibility of indicated C. glabrata strains. CSP was used at a concentration of 75 ng/ml (CSP-75) and 150 ng/ml (CSP-150) in CAA medium. C, representative confocal microscopy images of CAA medium–grown logarithmic-phase wt cells expressing CgSet4-GFP ectopically showing colocalization of CgSET4-GFP with the Hoechst 33258–stained nuclei. C. glabrata strains were grown in CAA medium lacking (CAA) or containing 64 μg/ml FLC or 150 ng/ml CSP for 1 h and stained with 1 μg/ml Hoechst 33258 for 15 min at 37 °C. Cells were washed, suspended in PBS, and visualized using the Confocal microscope (Zeiss LSM 700 equipped with 63×/1.44 numerical aperture objective). DIC, differential interference contrast; Hoechst 33258, DNA-binding stain. Bar represents 2.0 μm. D, qRT–PCR analysis showing transcriptional downregulation of the CgSET4 gene in response to FLC and CSP exposure. Log-phase wt cultures were left untreated or treated either with 64 μg/ml FLC or 250 ng/ml CSP for 1 h. RNA was isolated using the acid-phenol extraction method, and CgSET4 transcript levels were measured by qRT–PCR using the 2^−ΔΔCt method. Data (mean ± SEM, n = 3) were normalized against the CgTDH3 mRNA as control and represent fold change in CgSET4 gene expression in FLC– or CSP-treated wt cells, as compared with the CAA medium–grown wt cells (taken as 1.0). *p < 0.05; ***p < 0.001, paired two-tailed Student’s t test. E, representative immunoblots showing...
RNA-Seq analysis revealed 1077 genes to be differentially expressed (≥1.5-fold change in expression, and a q value of ≤0.05) in wt cells upon exposure to CSP (Fig. 4A and Table S2). Among these differentially expressed genes (DEGs), 475 and 602 genes were upregulated and downregulated, respectively (Fig. 4A and Table S2). Gene Ontology enrichment analysis for biological process by the DAVID (Database for Annotation, Visualization, and Integrated Discovery; https://david.ncifcrf.gov/) tool revealed upregulation of genes involved in fungal-type cell wall organization, response to oxidative stress, fatty acid (FA) beta-oxidation, and the catabolism of 5-carbon sugars, xylose and arabinose (Fig. 4B and Table S3A). Importantly, genes belonging to ergosterol biosynthesis, amino acid transport, zinc ion homeostasis, and amino acid biosynthetic process were found to be downregulated in CSP-treated wt cells, compared with untreated wt cells (Fig. 4B and Table S3A). Consistent with our qRT–PCR data (Fig. 2D), the CgSET4 gene was downregulated in response to CSP exposure (Table S3B). Of note, CSP treatment also led to the repression of master regulators of the ergosterol biosynthesis genes, CAGL0C01199g (CgUPC2A) and CAGL0F07865g (CgUPC2B), and the plasma membrane sterol transporter gene (CgAUS1), and upregulation of the negative regulator of ERG gene biosynthesis CAGL0D05434g (CgROX1), and glycosylphosphatidylinositol-anchored aspar-tyl protease genes (CgYPS1, CgYPS7, CgYPS6, and CgYPS10) (Table S2). These proteases have previously been implicated in maintenance of the cell wall integrity, with CgYps1 also contributing to CSP tolerance (42, 43), whereas CgRox1, a heme-dependent repressor of the hypoxic genes, has recently been implicated in negative regulation of the CgERG genes (44). Overall, our transcriptome data show an overlap with the previously reported transcriptomes of CSP-treated C. albicans and S. cerevisiae cells (41, 45, 46). For example, genes related to cell wall biogenesis and stress response, viz., BAG7, CHS1,
CWP1, FKS2, RLM1, and SLT2 were upregulated, and genes involved in amino acid biosynthesis, viz., ARG3, ILV6, and LEU1, were downregulated in both C. glabrata and S. cerevisiae upon CSP exposure (Table S2 and (41, 45)). Similarly, genes involved in ergosterol biosynthesis, ERG3, ERG4, ERG25, and ERG26, and high-affinity iron transport, FET3, FTR1, and FTH1, were downregulated in both C. glabrata and C. albicans (Table S2 and (46)). Furthermore, despite CgSet4 being a chromatin-associated protein, its loss did not have a big impact on the transcriptome of C. glabrata, and only 48 genes were found to be differentially expressed in the Cgset4Δ mutant, compared with
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Ergosterol is a major sterol in the plasma membrane and represents an integral cell membrane component, whose synthesis in the cell is regulated by oxygen and iron abundance (49, 50). Notably, ergosterol biosynthesis genes were upregulated in Cgset4Δ mutant (Table S4), with the mutant also exhibiting high level of resistance to both azole and echinocandin drugs (Fig. 1, A and B). In addition, CSP exposure resulted in downregulation of the ERG genes in wt cells (Table S2). Therefore, we hypothesized that besides impacting FLC resistance, ergosterol levels may also control resistance to the cell wall–targeting drug, CSP, in the Cgset4Δ mutant.

To test this hypothesis, we performed four experiments. First, we examined the expression of different ERG genes in the Cgset4Δ mutant. Ergosterol biosynthesis is a multistep process that consists of three modules (Fig. S7) (50). The mevalonate biosynthesis module takes place in the vacuole and the mitochondria, the farnesyl pyrophosphate biosynthesis pathway occurs in the vacuole, and the late stages of the pathway predominantly occur in the endoplasmic reticulum membrane (50, 51). The late pathway starts and ends with the formation of squalene and ergosterol, respectively (50, 51). Since late steps of the ergosterol biosynthesis pathway have been implicated in stress tolerance (50–52), we checked expression of the seven ERG genes, CgERG1, CgERG3, CgERG4, CgERG5, CgERG6, CgERG11, and CgERG25, which code for enzymes carrying out various reactions in the ergosterol biosynthesis pathway (Fig. S7). qRT–PCR analysis revealed about two- to five-fold higher expression of CgERG1, CgERG3, CgERG4, CgERG5, CgERG6, CgERG11, and CgERG25 genes in the Cgset4Δ mutant, compared with wt cells (Fig. 5A), suggesting that CgSET4 is required for repression of the late ERG genes under regular growth conditions. Of note, CgERG3, CgERG5, and CgERG11 transcript levels were also higher in Cgset4Δ mutant, compared with wt cells, in our RNA-Seq analysis (Table S4 and Fig. S6).

Cgset4 negatively regulates basal ERG gene expression

wt cells (Fig. 4C and Table S4). Of 48 genes, 24 were upregulated and 24 were downregulated in the Cgset4Δ mutant (Table S4). Of these gene sets, 7 and 10 genes were upregulated and downregulated, respectively, upon both deletion of CgSET4 gene and CSP treatment of wt cells (Tables S2 and S4). Furthermore, three genes were upregulated upon CSP treatment of wt cells, whereas these were downregulated upon CgSET4 deletion (Tables S2 and S4). These genes included CgARO10, CgHBT1, and CgHOR7 (Tables S2 and S4). Similarly, 12 genes were downregulated in response to CSP treatment of wt cells, whereas these were upregulated upon CgSET4 deletion, which also included four ergosterol biosynthesis genes, CgERG2, CgERG3, CgERG5, and CgERG11 (Tables S2 and S4). DAVID analysis revealed genes involved in ergosterol biosynthesis, sterol import, and carbohydrate transport to be upregulated (Table S5A), whereas genes involved in the inositol biosynthetic process were found to be downregulated, upon CgSET4 deletion in C. glabrata (Table S5B). Of note, CgERG2, CgERG3, CgERG5, CgERG11, CgAUS1, and CgROX1 were upregulated in Cgset4Δ cells, compared with wt cells (Table S4).

Notably, CSP exposure led to differential regulation of 1159 genes in the Cgset4Δ mutant, compared with untreated Cgset4Δ cells, with 568 and 591 displaying upregulation and downregulation, respectively (Fig. 4A and Table S6). Of these, 414 upregulated genes and 483 downregulated genes were common between CSP-treated wt and Cgset4Δ cells (Fig. 4A and Table S6). Gene Ontology analysis revealed a substantial overlap (898 genes) between CSP-treated wt and Cgset4Δ cells (Fig. 4C, Tables S3 and S7), with fungal-type cell wall organization, FA beta-oxidation, endocytosis, and protein unfold-
Second, we measured total ergosterol levels in the Cgset4Δ mutant. Consistent with increased ERG gene expression in the Cgset4Δ mutant, we found two-fold higher ergosterol content in the mutant, compared with wt cells (Fig. 5B). Expression of the CgSET4 gene led to wt-like ergosterol levels in the Cgset4Δ mutant (Fig. 5B), indicating that increased ergosterol content in the Cgset4Δ mutant was due to the lack of the CgSET4 gene. Collectively, these results suggest that CgSet4 inhibits the...
ergosterol biosynthesis process via downregulation of the CgERG genes in C. glabrata.

Third, we verified the effect of CgSET4 deletion on CSP-mediated downregulation of the ergosterol biosynthesis pathway, as RNA-Seq analysis had revealed ERG genes to be repressed in CSP-treated wt cells (Table S2 and Fig. S6). We observed that CSP treatment led to transcriptional repression of CgERG3, CgERG4, CgERG5, CgERG6, CgERG11, and CgERG25 genes in both wt (Fig. 5C) and Cgset4Δ (Fig. 5D) strains. Importantly, however, CgERG1 was found to be downregulated and upregulated in CSP-treated wt and Cgset4Δ cells, respectively (Fig. 5, C and D), indicating CgSET4-dependent transcriptional downregulation of the CgERG1 gene in response to CSP exposure. Of note, CgERG1 transcript levels were found to be increased upon CgSET4 deletion (Fig. 5A), suggesting a negative regulatory role for CgSET4 in CgERG1 expression. The molecular basis underlying CgSET4-dependent CSP-induced upregulation of CgERG1, which is the converse of that of the other CgERG genes, is yet to be determined. Altogether, our results suggest that CgSET4 is indispensable for the regulation of basal expression of ERG genes, but it has no prominent role in global downregulation of the ergosterol biosynthesis pathway under CSP-treated conditions.

Fourth, we measured ergosterol content in CSP-treated wt and Cgset4Δ cells. Contrary to expectations from the transcript profiling data, the CSP exposure did not result in a significant decrease in total ergosterol levels in wt cells (Fig. 5E). Instead, the ergosterol content was similar between untreated and CSP-treated wt cells (Fig. 5E). Similarly, ergosterol levels were similar between untreated and CSP-treated Cgset4Δ cells, with CgSET4 loss resulting in 2.0-fold to 2.5-fold higher ergosterol in both the presence and the absence of CSP (Fig. 5E). These results suggest a complex multifaceted regulation of the ergosterol biosynthesis process in response to the antifungal CSP, wherein the decreased transcript levels of ERG genes do not translate into lower cellular ergosterol levels. Alternatively, it is possible that while transcriptional downregulation of the ERG genes is observed within 1 h CSP treatment, changes in the cellular ergosterol levels may become conspicuous after prolonged CSP treatment when cells had undergone a few divisions. This possibility warrants further investigations. In this context, it is noteworthy that deletion of the CgUPC2A gene, which codes for a major transcriptional activator of ERG genes, was found to have no effect on the ergosterol content (44).

Deletion of CgERG3 and CgERG4 genes reverses FLC resistance in the Cgset4Δ mutant

The late ergosterol biosynthesis pathway enzymes have previously been implicated in stress tolerance in S. cerevisiae (50–52). CgERG3, CgERG5, and CgERG4 genes code for C-5 sterol desaturase, C-22 sterol desaturase, and C-24(28) sterol reductase enzymes, respectively, which catalyze last three steps of the ergosterol biosynthesis pathway (Fig. 5) (50). CgErg3, CgErg5, and CgErg4 produce ergosta-5,7,22,24(28)-tri-enol, ergosta-5,7,22,24(28)-tri-enol, and ergosterol, respectively (Fig. S7) (50, 51). Of note, the fungal Erg4 and Erg5 enzymes are not conserved in mammals (50), making them good antifungal targets. Importantly, mutations in the CgERG3 gene have recently been found in both FLC and anidulafungin (an echinocandin drug)-resistant isolates of C. glabrata (53). Since the Cgset4Δ mutant displayed resistance to FLC as well as to CSP (Fig. 1, A and D), we next undertook a genetic approach to decipher CSP-dependent modulation of the ergosterol biosynthesis pathway, which may have an impact on azole response. For this, we generated double deletion strains, which lacked both CgSET4, and CgERG3, CgERG5, or CgERG4 genes. As a control, the single deletion strains for CgERG3, CgERG4, and CgERG5 genes were also created. Growth analysis in the presence of FLC revealed that CgErg3 and CgErg4 are required for FLC tolerance, as Cgerg3Δ and Cgerg4Δ mutants displayed attenuated growth on FLC-supplemented medium (Fig. 6A). Of note, CgERG3 deletion has previously been associated with increased azole susceptibility (54). In contrast, CgERG5 gene loss had no effect on FLC susceptibility of C. glabrata (Fig. 6A). Ectopic expression of CgERG3 and CgERG4 genes complemented the elevated FLC susceptibility of Cgerg3Δ and Cgerg4Δ mutants, respectively (Fig. 6A), suggesting that the azole sensitivity is due to the lack of the corresponding CgERG gene. Furthermore, the deletion of CgERG3 and CgERG4 genes in the Cgset4Δ mutant background reversed the FLC resistance phenotype of the Cgset4Δ mutant (Fig. 6A), raising the possibility of an essential requirement for these genes as the downstream target/effect of CgSET4 dependent FLC response. Notably, similar to the Cgset4Δ mutant, the Cgset4Δcerg5Δ double mutant was found to be FLC resistant (Fig. 6A), indicating a dispensable role for CgErg5 in CgSet4-dependent modulation of the azole response. Moreover, since the Cgerg5Δ mutant did not exhibit elevated FLC susceptibility, it is possible that CgErg5 functions in ergosterol biosynthesis can either be bypassed and/or performed by another enzyme of the pathway. Alternatively, the differential FLC susceptibility of Cgerg3Δ, Cgerg4Δ, and Cgerg5Δ mutants could be due to impairment in both ergosterol biosynthesis and functioning of multidrug efflux pumps. In this context, it is noteworthy that the activity of multidrug transporter is known to be affected by the membrane lipid environment, and the plasma membranes of S. cerevisiae erg3Δ, erg4Δ, and erg5Δ mutants have been reported to be hyperpolarized, hyperpolarized, and non-hyperpolarized, respectively (55).

Deletion of CgERG5 reverses CSP resistance in the Cgset4Δ mutant

We next examined mutants’ growth in the presence of CSP. We found that while deletion of CgERG4 and CgERG5 genes rendered C. glabrata cells susceptible to CSP, CgERG3 gene loss led to CSP resistance (Fig. 6B). These results indicate opposite roles for CgERG3 and CgERG4 and CgERG5 genes in CSP susceptibility in C. glabrata. Of note, CgERG5 deletion has earlier been reported to result in CSP sensitivity (56).
Interestingly, CSP resistance in the Cgset4Δ mutant was not observed upon deletion of the CgERG5 gene, with the Cgset4Δerg5Δ mutant in fact displaying increased CSP susceptibility, compared with wt cells (Fig. 6B). Contrarily, the Cgset4Δerg3Δ and Cgset4Δerg4Δ mutants exhibited increased growth in the presence of CSP, similar to that of the Cgset4Δ mutant (Fig. 6B). These data point toward CgSet4-dependent and CgSet4-independent requirement of CgERG genes in...
regulating antifungal resistance, with CgERG4 loss resulting in increased susceptibility to bothazole and echinocandin tolerance, and CgERG3 and CgERG5 loss leading to increased FLC and decreased CSP, and increased CSP sensitivity, respectively. These data suggest that the ergosterol biosynthesis pathway may be more closely linked with the action of cell wall–targeting echinocandin drugs, than it is currently being considered. In this context, it is worth noting that Cgerg1Δ, Cgerg3Δ, and Cgerg11A mutants have been shown to exhibit altered expression of ERG genes (23, 54). Therefore, it is possible that the differential expression of other ERG genes may contribute to distinct antifungal susceptibilities of double deletion mutants. However, the precise basis underlying the differential CSP susceptibilities of CgergΔ and Cgset4ΔergΔ mutants is yet to be determined.

Altogether, we infer from these data that the upregulated ergosterol biosynthesis process in the Cgset4Δ mutant contributes to elevated ergosterol content and may also modulate the response to CSP stress. Our data also raise a possibility of a possible nexus between CgSet4-dependentazole and echinocandin resistance and CgSet4-dependent negative regulation of CgERG gene expression.

**CgSet4 regulates ergosterol biosynthesis through CgUpc2a**

To investigate the molecular link between CgSet4-mediated repression of CgERG genes and CSP resistance, we focused on two genes, CgUPC2A and CgUPC2B, that code for two Zn2+-Cys6 transcriptional activators of the ergosterol biosynthesis pathway in _C. glabrata_ (21), and performed four experiments. First, we checked transcript levels of CgUPC2A and CgUPC2B genes in the Cgset4Δ mutant and found 2.3-fold higher and similar CgUPC2A and CgUPC2B gene expression, respectively, in the Cgset4Δ mutant, compared with wt cells (Fig. 7A). Furthermore, CSP exposure led to 2.4-fold and 1.5-fold downregulation of CgUPC2A and CgUPC2B genes, respectively, in wt cells (Fig. 7A). Contrarily, CgUPC2A expression remained the same between CSP-treated and untreated Cgset4Δ cells (Fig. 7A), suggesting that CgSet4 may be required to control CgUPC2A gene expression in response to CSP exposure. Of note, a small 1.4-fold decrease in CgUPC2B transcript levels was observed in CSP-treated Cgset4Δ cells, compared with untreated Cgset4Δ cells (difference not conspicuous in Figure 7A, as it depicts comparison with the untreated wt cells), thereby ruling out a major role of CgSet4 in CSP-induced CgUPC2B downregulation.

Second, we created deletion strains for CgUPC2A and CgUPC2B genes in both wt and Cgset4Δ strain backgrounds. Deletion of the CgUPC2B gene had no impact on the susceptibility toward FLC (Fig. 7B) or CSP (Fig. 7C). Contrarily, CgUPC2A gene loss rendered cells highly and moderately sensitive to FLC (Fig. 7B) and CSP (Figs. 7C and 58), respectively. These FLC susceptibility data are consistent with the published reports of CgUpc2a being a major regulator of ergosterol biosynthesis genes in _C. glabrata_ under normal laboratory-growth conditions (21, 22). CgUpc2a has also been shown to be required for basal and FLC-induced transcription of ERG genes, with CgUPC2A deletion in anazole-susceptible dose-dependent and an azole-resistant clinical isolate leading to decreased ergosterol content (22). Of note, CgUPC2A gene loss has recently also been associated with CSP sensitivity (57).

Notably, the double mutant Cgset4Δupc2aΔ exhibited resistance to both FLC and CSP drugs, similar to the Cgset4Δ mutant (Fig. 7, B and C). Contrary to this, the Cgset4Δupc2aΔ double mutant was found to be sensitive to both antifungals, compared with the Cgset4Δ mutant (Fig. 7, B and C). Importantly, the Cgset4Δupc2aΔ double mutant displayed highly attenuated growth in rich yeast extract–peptone–dextrose (YPD) medium (Fig. 59), underscoring the stress that the simultaneous loss of both genes poses to the cellular machinery. Importantly, ectopic expression of CgSET4, CgUPC2A, and CgUPC2B genes in single and double mutants complemented the mutants’ altered drug susceptibility phenotypes (Fig. 7, B and C); however, the phenotypes were not rescued fully in Cgset4Δupc2aΔ and Cgset4Δupc2Δ mutants expressing CgUPC2A and CgUPC2B, respectively (Fig. 7, B and C). The basis underlying this observation is yet to be determined.

Based on these data, which suggest that CgSet4 is likely to control ergosterol biosynthesis process by modulating the CgUpc2A-dependent ERG gene regulation, we hypothesized that the elevated expression of CgUPC2A and its target genes contribute to antifungal resistance in the Cgset4Δ mutant. If this is true, the CgUPC2A gene deletion is likely to impact the antifungal resistance gene expression negatively in the Cgset4Δ mutant. Therefore, to test this hypothesis, we checked the expression of CgPDR1, CgERG3, and CgERG11 genes in the Cgset4Δupc2aΔ double mutant. We found two-fold lower, similar, and similar transcript levels of CgPDR1, CgERG3, and CgERG11 genes, respectively, in the double mutant, compared with wt cells (Fig. 7D), indicating that CgUpc2A is a major activator of the CgPDR1 gene in the Cgset4Δ mutant. Furthermore, the wt-like expression of CgERG3 and CgERG11 genes in the Cgset4Δupc2aΔ double mutant, in comparison to elevated CgERG3 and CgERG11 transcript levels in the Cgset4Δ mutant (Fig. 5A), implicates CgUpc2A in the activation of CgERG genes in the Cgset4Δ mutant.

Finally, to examine if CgSet4 directly regulates CgUPC2A expression, we performed the chromatin immunoprecipitation (ChIP) analysis using CgSet4-GFP, and found CgSet4 bound to the CgUPC2A promoter. Furthermore, CSP exposure led to a decrease in the occupancy of CgSet4 on the CgUPC2A promoter (Fig. 7E), which is consistent with a reduction in CgSET4 transcript and protein levels in response to CSP exposure (Fig. 2, C and D). These data indicate multifactorial regulation of CgUPC2A gene expression, with probably another repressor accounting for the CSP-induced CgUPC2A downregulation. Notably, Cg Rox1 has recently been reported to be a negative regulator of ERG genes, with CgRox1Δupc2aΔ mutant containing 1.5-fold higher ergosterol levels, indicating perturbed sterol homeostasis (44). Based on this report and the following three findings of our RNA-Seq analysis, we speculate that CgRox1 could play a role in CgUPC2A downregulation upon CSP exposure. First, the Cgset4Δ mutant was found to be...
Figure 7. CgUpc2A regulates caspofungin (CSP) susceptibility of the Cgset4Δ mutant.

A, qRT–PCR analysis showing transcriptional downregulation of CgUPC2A and CgUPC2B genes in response to CSP exposure. Log-phase wt and Cgset4Δ cells were left untreated or treated with 250 ng/ml CSP in CAA medium for 1 h, and CgUPC2A and CgUPC2B transcript levels were determined by qRT–PCR. Data (mean ± SEM, n = 3) were normalized against the CgTDH3 mRNA control and represent fold change in gene expression in indicated strains as compared with CAA medium–grown wt cells (taken as 1.0). *p < 0.05; **p < 0.01, paired two-tailed Student’s t test.

B, serial dilution spotting analysis illustrating fluconazole susceptibility of indicated C. glabrata strains. Fluconazole was used at a concentration of 16 μg/ml (FLC-16), 32 μg/ml (FLC-32), and 64 μg/ml (FLC-64) in CAA medium.

C, liquid medium–based growth analysis illustrating CSP susceptibility of indicated C. glabrata strains. C. glabrata strains were cultured at 30 °C in CAA medium lacking (CAA) or containing 75 ng/ml (CSP-75) or 150 ng/ml (CSP-150) CSP for 16 h. After incubation, cultures were diluted in PBS, and 3 μl of undiluted, and 10-, 100-, and 300-fold diluted cultures were spotted on CAA medium, and growth was recorded after 1 day of growth at 30 °C. D, qRT–PCR analysis showing CgPDR1, CgERG3, and CgERG11 transcript levels in Cgset4Δupc2aΔ mutant. Transcript levels were measured in YPD medium–grown, log-phase wt, and Cgset4Δupc2aΔ cells by qRT–PCR. Data (mean ± SEM, n = 3–4) were normalized against the CgTDH3 mRNA control, and represent fold change in gene expression in the double mutant Cgset4Δupc2aΔ as compared with wt cells (taken as 1.0). **p < 0.01, paired two-tailed Student’s t test. E, ChIP analysis, showing CgSet4-GFP enrichment on CgUPC2A promoter (5’UTR) under normal growth conditions and decreased enrichment upon CSP exposure, was performed with anti-GFP antibody to detect CgSet4-GFP. Log phase–grown Cgset4Δ/Vector and Cgset4Δ/CgSET4-GFP strains were either left untreated or treated with
Role of CgSet4 in antifungal drug resistance

C. glabrata is a common cause of Candida bloodstream infections in patients with a weakened immune system (1). The successful treatment of C. glabrata infections is becoming increasingly difficult as two mainstream antifungal drugs, azoles and echinocandins, are proving to be less effective because of emerging resistance in C. glabrata isolates in hospitals (4, 5, 11, 12). Not only C. glabrata is intrinsically less susceptible to frequently used, cost-effective, and relatively safeazole antifungals, but it also acquires high levels of resistance to azole and echinocandin drugs, with the echinocandin-cassette representing the last line of antifungal therapy in many hospitalized severely ill patients (4, 5, 9, 11, 12, 14, 15). Theazole resistance in clinical settings has predominantly been attributed to gain-of-function mutations in the master transcriptional regulator-encoding gene, CgPDR1, whereas mutations in the hot-spot regions of β-1,3 glucan synthase enzyme–encoding genes CgFKS1 and CgFKS2 primarily account for echinocandin resistance in hospitals worldwide (14, 15). Chromatin architecture is pivotal to gene expression regulation, with histone post-translational modifications playing a key role in maintenance of chromatin homeostasis (58). The epigenetic control of antifungal resistance mechanisms in C. glabrata is beginning to be elucidated (28, 38, 56).

Toward this end, we report an essential role for the SET domain–containing protein CgSet4 in the transcriptional downregulation of CgPDR1 and CgUPC2A genes, which code for the master regulator of MDR and ergosterol biosynthesis genes, respectively, in C. glabrata. In addition, we present the first systematic analysis unveiling functions of CgSet1–CgSet6 and CgErg3–CgErg5 proteins in C. glabrata. We show that the last enzyme of the ergosterol synthesis pathway CgErg4 is required for bothazole and echinocandin tolerance, and, of six SET domain–containing proteins, only CgSet4 acts as a negative regulator of bothazole and echinocandin antifungal resistance.

The SET domain, that consists of strongly conserved sequence motif of about 130 amino acids, was initially identified in the Drosophila proteins, Su(var)3 to 9, Enhancer-of-ozeste and Trithorax, which regulate gene expression during development (35, 59). The SET domain now has been reported in several proteins that perform diverse functions (30, 36, 37).

The National Center for Biotechnology Information conserved domain search analysis (https://www.ncbi.nlm.nih.gov/Structure/cdd/wrpsb.cgi) revealed that the SET domain in CgSet4 belongs to the SET Superfamily cl40432. The C. glabrata Set4 protein is 350 amino acid-long, whereas Set4 in S. cerevisiae consists of 560 amino acids. Despite sharing 40% identity with CgSet4 protein, the S. cerevisiae Set4 could not complement the decreased FLC and CSP susceptibility of the Cgset4Δ mutant (Fig. 2, A and B). In addition, while CgSET4 expression was downregulated (Fig. 2D), ScSET4 transcription was found to be activated upon azole exposure (33). These results highlight functional differences between the two proteins and are in accordance with other reported functions of ScSet4 protein. For example, ScSet4 plays an essential role in protection against oxidative stress by activating stress response genes (31), and 196 genes were found to be differentially regulated upon ScSET4 disruption (34). Contrarily, CgSET4 deletion had a minor effect on the C. glabrata transcriptome, with 48 genes displaying deregulation (Table S4). Moreover, CgSET4 deletion did not lead to an increased susceptibility toward hydrogen peroxide–induced oxidative stress (Fig. 1B), which could in part be due to elevated expression of the CgCTA1 gene in the Cgset4Δ mutant (Table S4). Furthermore, unlike ScSET4 (31), ectopic expression of CgSET4 was not found to be detrimental for cell growth in C. glabrata (Fig. 2A).

250 ng/ml CSP in CAA medium for 1 h. The percentage of input was calculated for each IP and the ChIP amplification was normalized to the DNA input samples. Data (n = 2) represent CgSet4 occupancy in both untreated and drug-treated conditions, compared with Cgset4Δ/Vector samples. The primers used detected the promoter and the internal region of the CgUPC2A gene. **p < 0.01, paired two-tailed Student’s t test. CAA, casamino acid; ChIP, chromatin immunoprecipitation; IP, immunoprecipitation; qRT–PCR, quantitative RT–PCR.

Discussing antifungal drug resistance

Discussion

C. glabrata is a common cause of Candida bloodstream infections in patients with a weakened immune system (1). The successful treatment of C. glabrata infections is becoming increasingly difficult as two mainstream antifungal drugs, azoles and echinocandins, are proving to be less effective because of emerging resistance in C. glabrata isolates in hospitals (4, 5, 11, 12). Not only C. glabrata is intrinsically less susceptible to frequently used, cost-effective, and relatively safeazole antifungals, but it also acquires high levels of resistance to azole and echinocandin drugs, with the echinocandin-cassette representing the last line of antifungal therapy in many hospitalized severely ill patients (4, 5, 9, 11, 12, 14, 15). Theazole resistance in clinical settings has predominantly been attributed to gain-of-function mutations in the master transcriptional regulator-encoding gene, CgPDR1, whereas mutations in the hot-spot regions of β-1,3 glucan synthase enzyme–encoding genes CgFKS1 and CgFKS2 primarily account for echinocandin resistance in hospitals worldwide (14, 15). Chromatin architecture is pivotal to gene expression regulation, with histone post-translational modifications playing a key role in maintenance of chromatin homeostasis (58). The epigenetic control of antifungal resistance mechanisms in C. glabrata is beginning to be elucidated (28, 38, 56).

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250 ng/ml CSP in CAA medium for 1 h. The percentage of input was calculated for each IP and the ChIP amplification was normalized to the DNA input samples. Data (n = 2) represent CgSet4 occupancy in both untreated and drug-treated conditions, compared with Cgset4Δ/Vector samples. The primers used detected the promoter and the internal region of the CgUPC2A gene. **p < 0.01, paired two-tailed Student’s t test. CAA, casamino acid; ChIP, chromatin immunoprecipitation; IP, immunoprecipitation; qRT–PCR, quantitative RT–PCR.
Role of CgSet4 in antifungal drug resistance

Despite these differences, Set4 in both C. glabrata and S. cerevisiae acts as a negative regulator of ergosterol biosynthesis (ERG) genes. SET4 disruption led to azole resistance, with Set4 regulating ERG3 and ERG11 expression through direct binding to their promoters in S. cerevisiae (33). Ergosterol biosynthesis in fungi is a multistep energy-consuming process, with oxygen and heme acting as cofactors for many enzymes of the ergosterol synthesis pathway (Fig. S7) (50, 51). Furthermore, because of the centrality of ergosterol in maintaining fluidity, integrity, and functions of the plasma membrane, ergosterol biosynthesis is regulated at multiple levels, including transcriptional regulation, transport, and sterol feedback inhibition, and subcellular localization of enzymes (50, 51). The late steps of the ergosterol biosynthesis pathway involve many demethylation, reduction, and desaturation reactions (Fig. S7), with ergosterol being transported to the plasma membrane from the site of its synthesis, endoplasmic reticulum (50, 51).

ERG gene expression is tightly regulated at the transcriptional level, with hypoxia and iron depletion resulting in downregulation of ergosterol synthesis in S. cerevisiae (31, 33, 49). Azole antifungals target a rate-limiting step of the enzyme, which is encoded by the ERG11 gene (13, 14). Azole exposure in C. glabrata is known to result in elevated expression of ERG genes including ERG11, and this gene induction is largely carried out by the Zn2-Cys6 binuclear cluster transcription factor, CgpUpc2a (21–23, 60). We show that CgSET4 deletion led to the increased expression of CgUPC2A, along with its target ERG genes, viz., CgERG2, CgERG3, CgERG4, CgERG6, and CgERG11 (Figs. 5A and 7A). In addition, elevated transcript levels of the zinc finger transcriptional activator gene CgPDR1 as well as the multidrug transporter genes CgCDR1 and CgCDR2 (CgPdr1 target genes) in the Cgset4Δ mutant (Fig. 3A) suggest that CgSet4 is a general repressor of two major azole response pathways in C. glabrata. Consistent with this, ChIP analysis revealed CgSet4-GFP to be present at the promoter region of CgPDR1 (Fig. S10). Of note, the CgSet1-dependent H3K4 methylation has recently been found to be increased on actively transcribing ERG genes in response to FLC (38), thereby highlighting the epigenetic regulation of CgERG genes in C. glabrata.

In S. cerevisiae, the major sterol regulator Upc2a, a homodimer, is known to bind to the 7 bp sterol regulatory element (SRE) sequence TATAACA that is present in promoters of the ERG, sterol uptake, the DAN/TIR genes (50, 51). The C-terminal domain of Upc2 has been shown to act as an ergosterol-binding and sensing domain, with the ergosterol-bound Upc2 residing in the cytosol (50). Ergosterol depletion leads to the release of ergosterol, and the translocation of Upc2a to the nucleus, resulting in the transcriptional activation of its targets including ERG genes (50). The C. glabrata ortholog of S. cerevisiae Upc2, CgUpc2a, has recently been postulated to act like its S. cerevisiae counterpart and shown to regulate the expression of a vast array of genes including ERG and CgPDR1 regulon genes, which contain SREs in their promoter regions (57).

Through ChIP-Seq analysis, Vu et al. (57) showed that CgUpc2a binds to about 1000 genes in C. glabrata. In addition, 64 genes including CgERG4 were identified as indirect target genes of CgUpc2a (57). CgUPC2A itself was found to be upregulated upon FLC treatment, and its disruption rendered cells susceptible to both FLC and CSP drugs (57). In addition, although FLC-induced upregulation of CgCDR1 and CgPDR1 genes was lower and similar between wt and Cgupc2aΔ strains, respectively (57), a pivotal role for CgUpc2a in CgPdr1-dependent gene network was unveiled by the reduced binding of CgUpc2a to the mutated SRE in the CgPDR1 promoter, as well as, by the diminished FLC-induced induction of the SRE-lacking CgPDR1 gene (57). Our results of reduced CgPDR1 gene expression in the double mutant Cgset4Δuppc2aΔ (Fig. 7D) suggest that the elevated CgUPC2A transcript levels contribute to the increased CgPDR1 gene expression in the Cgset4Δ mutant. Of note, further detailed investigations are required to determine if CSP susceptibility of the Cgset4Δuppc2aΔ mutant is due to an imbalance of sterol species or cell wall components or both.

In addition to the SET proteins, we have also investigated the role of three enzymes catalyzing late stages of ergosterol synthesis, CgErG3, CgErG5, and CgErG4, in antifungal tolerance and virulence. Intriguingly, while the Cgerg3Δ and Cgerg4Δ mutants exhibited elevated FLC sensitivity, the Cgerg5Δ mutant displayed FLC susceptibility, similar to that of the wt strain (Fig. 6, A and C). Furthermore, deletion of the CgErG3, CgErG5, and CgErG4 genes led to decreased, increased, and increased susceptibility to CSP, respectively (Fig. 6B). Notably, the echinocandin-resistant isolates in a microevolution study, which exhibited crossresistance to FLC, have recently been shown to carry mutations in the CgErG3 gene (53). Our mice infection studies revealed that in line with their distinct role in antifungal resistance, CgErG3 and CgErG4 were required for survival of C. glabrata in the murine model of systemic candidiasis in an organ-dependent manner, whereas CgErG5 was dispensable in this model (Fig. S11). In addition, whereas CgUPC2A deletion led to significantly attenuated survival of C. glabrata in various organs, CgUPC2B deletion affected survival adversely only in kidneys (Fig. S11), indicating that CgUpc2a plays a major role in virulence of C. glabrata. These results together also highlight the importance of ergosterol synthesis for C. glabrata–mammalian host interaction.

Furthermore, our data underscore that CgErg enzymes differ from one another in their requirement in cellular response to FLC and CSP. This difference among CgErg enzymes is likely to be determined by additional regulatory factors and/or other functions of CgErg proteins. These results are consistent with varied regulatory mechanisms of different ERG genes in S. cerevisiae and C. glabrata (21, 23, 33, 50, 57, 60). In this context, it is worth noting that despite CgUpc2a showing strong binding to the CgERG1 gene promoter, FLC-induced activation of CgERG1 gene was found to be similar between wt and Cgupc2aΔ mutant (57), thereby pointing toward the complex multifactorial environmental cue-dependent
regulation of individual CgERG genes. Our data provide further support to this notion, as CgSet4 was found to be indispensable and dispensable for the CSP-induced repression of CgERG1, and other CgERG (CgERG4, CgERG5, CgERG6, CgERG11, and CgERG25) genes, respectively (Fig. 5, C and D).

CSP exposure has recently been reported to result in elevated reactive oxygen species production (61). Consistent with this, our transcriptional profiling analysis revealed upregulation of oxidative stress response genes in CSP-treated cells. Furthermore, a multifactorial role of mitochondria has recently been reported in CSP tolerance in C. glabrata (61). Given that a reduction in ergosterol content is known to adversely affect mitochondrial DNA maintenance (62), it is possible that elevated ergosterol in Cgset4Δ mutant modulates mitochondrial functions, which may contribute to CSP resistance in the mutant. Elucidation of the nexus among sterol metabolism, CSP resistance, and mitochondrial functions will shed light on the underlying molecular mechanism.

Collectively, our data suggest that the transcriptional regulation of ERG genes is not necessarily reflected in cellular ergosterol levels. Consistently, despite CgUpc2a regulating ERG gene expression, its disruption led to no significant decrease in ergosterol levels (44, 57). Similarly, cellular ergosterol levels were found to be decreased upon loss of both UPC2 and ECM22 (paralog of UPC2) genes, which code for activators of the sterol biosynthetic pathway in S. cerevisiae (50, 63). Based on our data, we propose that CgSet4 is a negative regulator of the basal-level expression of the CgUPC2A gene (Fig. 8), whereas the cellular response to CSP involves a wholesale downregulation of ergosterol biosynthesis pathway that is probably initiated by the transcriptional downregulation of CgUPC2A (Fig. 8). CSP-induced repression of CgUPC2A is probably primarily carried out by another repressor protein (Fig. 8), with CgRox1 to be a likely candidate whose expression was found to be upregulated in response to CSP exposure (Table S2). Of note, CgRox1 has recently been shown to be a negative regulator of ERG genes, with loss-of-function mutations in CgROX1 rescuing the increased FLC susceptibility of the Cgupc2aΔ mutant (44). Furthermore, since theazole and CSP resistance of the Cgset4Δ mutant is reversed by CgUPC2A deletion (Fig. 7, B and C), CgUpc2a is likely to be the main effector protein of CgSet4, with CgUpc2a also

**Role of CgSet4 in antifungal drug resistance**

Candida glabrata cells maintain cellular ergosterol levels via tight regulation of ergosterol biosynthesis (CgERG) genes. CgSet4 acts as a repressor of CgPdr1-dependent multidrug resistance, and ergosterol biosynthesis pathways, as CgSET4 deletion leads to increased basal expression of CgPDR1 regulon and CgERG genes, elevated ergosterol content, and resistance to fluconazole and CSP antifungals. Under regular growth conditions, CgSet4 keeps CgUPC2A expression in check via binding to the CgUPC2A promoter. Since CgUpc2a is an activator of CgERG and CgPDR1 genes, the lower levels of CgUpc2a result in restrained expression of CgERG and CgPDR1 genes, thereby maintaining sterol homeostasis and susceptibility to azole antifungals. CSP exposure results in transcriptional and post-transcriptional downregulation of CgSET4, reduction in CgSet4 abundance on the CgUPC2A promoter, transcriptional activation and repression of CgROX1 and CgUPC2A, respectively, and the consequent downregulation of CgERG genes. Two lines of evidence, the Cgset4Δ mutant’s proficiency in downregulating CgERG genes upon CSP exposure, and the decreased CgSet4 occupancy on CgUPC2A promoter in CSP-treated wt cells, point toward another repressor protein (repressor X) contributing to CgUPC2A and CgERG gene downregulation in response to CSP. This repressor is yet to be identified, although CgRox1, being an inhibitor of CgERG gene expression, appears to be a strong candidate for the same.
controlling expression of the CgPdr1 regulon genes. Although the molecular basis for CgSet4-dependent regulation of CgPdr1 and CgUpc2a regulon genes is yet to be deciphered, our ChIP data suggest that it is likely to be through direct association of CgSet4 with the regulatory regions of CgUpc2A. However, the regulatory region of CgUpc2a, where CgSet4 could bind to, is yet to be identified.

Importantly, owing to a general repressive effect of CSP on ERG gene expression, our data underscore the need to revisit the combinatorial therapeutic regimen, which involves treatment of fungal infections with echinocandins along with other ergosterol-targeting drugs, polyenes (bind to ergosterol in the cell membrane), allylamines (inhibit squalene epoxidase, encoded by ERG1), or azoles (64, 65).

Altogether, our data demonstrate CgSet4 to be a key regulator of CgPdr1-dependent MDR and CgUpc2a-dependent ergosterol biosynthesis pathways, thereby making CgSet4 as a major component of the cell wall and cell membrane homeostasis systems in C. glabrata.

Experimental procedures

Strains, media, and growth

C. glabrata strains used in the study were derivatives of the BG2 strain and maintained in the rich YPD or minimal casamino acid (CAA) medium at 30 °C. The Escherichia coli DH5-α strain was used for plasmid propagation and maintained in LB medium at 37 °C. Overnight cultures of C. glabrata strains were grown for 3 to 4 h at 30 °C to obtain log-phase cultures. Antifungal drug and stress susceptibility was examined by serial dilution spotting assay and liquid medium–based growth analysis.

C. glabrata gene deletion and cloning

The homologous recombination–based strategy was used to create C. glabrata single and double deletion strains using the nat1 gene, which confers nourseothricin resistance, as a recyclable selection marker, as described previously (66). The Cgset4Δ strain was used as the parental strain to generate double mutants, Cgset4Δcdr1Δ, Cgset4Δerg3Δ, Cgset4Δerg4Δ, Cgset4Δerg5Δ, Cgset4Δupc2aΔ, and Cgset4Δupc2bΔ. For overexpression studies, CgSET4 (CAGL0G04499g, 1.05 kb) gene was cloned under the strong PDC1 promoter in the pRK1349 plasmid. For complementation studies, CgSET4 gene was cloned with its own promoter (1 kb region upstream of the start codon ATG) in the pGRB2.1 plasmid at XbaI and XmaI restriction enzyme sites. The ergosterol biosynthesis genes CgERG3 (CAGL0F01793g, 3.1 kb), CgERG4 (CAGL0A00429g, 3.4 kb), CgERG5 (CAGL0M07656g, 3.6 kb), CgUPC2a (CAGL0C01199g, 4.7 kb), and CgUPC2b (CAGL0F07865g, 4.5 kb) were cloned at XbaI–Xmal, Xhol–Xmal, Spe–Xmal, Xbal–Xmal, and Spe–Xmal restriction enzyme sites, respectively, in the pGRB2.2 plasmid. For generation of CgSet4-GFP construct, the CgSET4 ORF (without the stop codon) was cloned downstream and upstream of the PGK1 promoter and GFP-encoding region, respectively, at Spe–Xmal restriction enzyme sites in the pGRB2.3 plasmid. The strains, plasmids, and primers used in this study are listed in Tables S8–S10, respectively.

Protein extraction and immunoblotting

For protein expression studies, log-phase C. glabrata cells were collected and washed with PBS. Cells were lysed in the lysis buffer (20 mM Tris–HCl [pH 8.0], 100 mM NaCl, 1 mM EDTA, with 1 mM sodium orthovanadate, 1 mM PMSF, 10 mM sodium fluoride, and 1× protease inhibitor) mechanically using glass beads. After centrifugation of lysates for 15 min at 15,000 rpm at 4 °C, the supernatant was collected and resolved on SDS-PAGE. The proteins were transferred to the polyvinylidene difluoride membrane and probed with anti-H3K4me3 (Abcam; catalog no.: ab8580), anti-H3K36me3 (Abcam; catalog no.: ab9050), anti-histone H3 (Abcam; catalog no.: ab1791), anti-GFP (Abcam; catalog no.: ab290), or anti-Gapdh (Abcam; catalog no.: ab22555) antibodies.

Mice infection assay

Mice infection studies were performed at the Animal House Facility of Centre for DNA Fingerprinting and Diagnostics (CDFD), Hyderabad, India in accordance with guidelines of the Committee for the Purpose of Control and Supervision of Experiments on Animals, Government of India, and were approved by the Institutional Animal Ethics Committee [EAF/RK/CDFD/15]. Briefly, C. glabrata strains were grown overnight in YPD or CAA medium, washed with PBS, and suspended in PBS. C. glabrata cells (4 × 10⁷; 100 μl PBS cell suspension) were injected into the tail vein of 6- to 8-week-old female BALB/c mice. Mice were monitored for 7 days, sacrificed, and four organs, kidneys, liver, brain, and spleen, were collected. Organs were homogenized in PBS, and appropriate dilutions were plated on penicillin- and streptomycin-containing YPD medium. Mouse organ fungal burden was determined by counting colonies manually.

qRT–PCR

Total RNA was extracted from appropriate strains using the acid-phenol extraction method and digested with DNase I to eliminate any DNA contamination. DNase I-digested RNA (500 ng) was used to synthesize complementary DNA using the SuperScript III First-Strand Synthesis System for RT–PCR. The qPCR was performed using the SYBR green real-time PCR mastermix, and the sample C_T (cycle threshold) values were normalized against the C_T value of the control house-keeping CgACT1 or CgTDH3 gene. The fold change in expression under different conditions was determined using the comparative C_T (2^-ΔΔC_T) method.

Microscopy analysis

The overnight grown Cgset4Δ/CgSET4-GFP strain was grown to log phase in CAA medium, followed by growth either in the absence (CAA) or the presence of FLC (64 μg/ml) or CSP (150 ng/ml), for 1 h. After incubation, cells corresponding to absorbance of 1.0 at 600 nm were collected, washed with PBS, and suspended in 100 μl of PBS containing 1 μg/ml...
incubated at 85°C for 3 h. The samples were kept static for about 15 min for phase separation, beads were given four consecutive washes, with FA alcohol, suspended in TE, treated with RNAse at 37°C, and incubated overnight at 65°C for decrosslinking, followed by proteinase K treatment for 1 h. DNA was precipitated using phenol:chloroform:isoamyl alcohol, suspended in TE, treated with RNAses at 37°C for 1 h, and used as template for qRT–PCR using appropriate set of primers.

Other procedures
The cell wall analysis was performed, as described previously (66).

Statistical and functional analysis
The statistical significance was determined using the GraphPad Prism software (GraphPad Software, Inc). The two-tailed Student’s t test and the non-parametric Mann–Whitney test were used for intergroup comparisons and mouse organ fungal burden analysis, respectively. DEGs were analyzed and functionally annotated using the Candida Genome Database (http://www.candidagenome.org) and DAVID (https://david.ncifcrf.gov/) tools.

Data availability
The raw RNA-Seq data have been submitted to the National Center for Biotechnology Information Gene Expression Omnibus repository (https://www.ncbi.nlm.nih.gov/geo/), with Gene Expression Omnibus accession number GSE202654.

Supporting information—This article contains supporting information (27, 30, 68–71).

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Conflict of interest—The authors declare that they have no conflicts of interest with the contents of this article.

Abbreviations—The abbreviations used are: CAA, casamino acid; CDFD, Centre for DNA Fingerprinting and Diagnostics; ChiP, chromatin immunoprecipitation; CSP, caspofungin; C_T, cycle threshold; DAVID, Database for Annotation, Visualization, and Integrated Discovery; DEG, differentially expressed gene; DHE, dehydroergosterol; FA, fatty acid; FLC, fluconazole; MDR, multidrug resistance; qRT–PCR, quantitative RT–PCR; SET, [suppressor of varieation 3 to 9 [Su(var)3–9], enhancer of zeste [E(z)], and trithorax (Trx)]; SRE, sterol regulatory element; YPD, yeast extract–peptone–dextrose.

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