The yeast pantothenate kinase Cab1 is a master regulator of sterol metabolism and of susceptibility to ergosterol biosynthesis inhibitors

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Joy E. Chiu, Jose Thekkiniath, and Choukri Ben Mamoun are listed on a patent application for targeting pantothenate kinase (PanK) activity for development of antifungal drugs. Choukri Ben Mamoun is the founder of a company that aims to develop such drugs. This article contains Figs. S1 and S2.

In fungi, ergosterol is an essential component of the plasma membrane. Its biosynthesis from acetyl-CoA is the primary target of the most commonly used antifungal drugs. Here, we show that the pantothenate kinase Cab1p, which catalyzes the first step in the metabolism of pantothenic acid for CoA biosynthesis in budding yeast (Saccharomyces cerevisiae), significantly regulates the levels of sterol intermediates and the activities of ergosterol biosynthesis-targeting antifungals. Using genetic and pharmacological analyses, we show that altered pantothenate utilization dramatically alters the susceptibility of yeast cells to ergosterol biosynthesis inhibitors. Genome-wide transcription and MS-based analyses revealed that this regulation is mediated by changes both in the expression of ergosterol biosynthesis genes and in the levels of sterol intermediates. Consistent with these findings, drug interaction experiments indicated that inhibition of pantothenic acid utilization synergizes with the activity of the ergosterol molecule—targeting antifungal amphotericin B and antagonizes that of the ergosterol pathway—targeting antifungal drug terbinafine. Our finding that CoA metabolism controls ergosterol biosynthesis and susceptibility to antifungals could set the stage for the development of new strategies to manage fungal infections and to modulate the potency of current drugs against drug-sensitive and -resistant fungal pathogens.

Fungal diseases are a major global health problem and are particularly threatening as opportunistic infections in immunosuppressed individuals such as AIDS and cancer patients (1, 2). Despite major advances in understanding the biology of fungi and in antifungal drug discovery, fungal infections continue to cause significant mortality and morbidity worldwide (3). Commonly used antifungal drugs include azoles, echinocandins, polyenes, and allylamines (4). However, due to widespread spread resistance to some of these drugs and their lack of efficacy against a diverse array of pathogens such as Candida albicans, Candida auris, and Aspergillus fumigatus, both new drugs and alternative strategies to modulate the efficacy and safety of current drugs and reverse resistance are urgently needed.

In all living organisms, coenzyme A (CoA) plays a fundamental role in cellular metabolism. Fig. 1 shows a simplified schematic of metabolic pathways for CoA biosynthesis in yeast, whereas Fig. 2 shows more comprehensive detail. The unique chemical structure of CoA, with a reactive thiol group and a nucleotide moiety, allows it to serve as a cofactor in critical biochemical and regulatory functions through activation of carboxylic acids and production of thioester derivatives (5). In the yeast Saccharomyces cerevisiae, CoA is synthesized from vitamin B₆ (pantothenic acid) either imported into the cell via the Fep2p pantothenate transporter or produced de novo from the ligation of β-alanine and pantoate by the pantothenate synthase Pan6p (6, 7) (Figs. 1 and 2). Pantothenic acid utilization is absolutely essential for yeast survival as a double mutant lacking both the FEN2 and PAN6 genes is inviable (8). The first step in pantothenate utilization is its phosphorylation by the pantothenate kinase Cab1p, encoded by a single-copy gene, CAB1 (8). This gene is required for yeast viability as deletion of CAB1 results in cell death. A mutant, cab1Δ, which produces a kinase enzyme with glycine 351 substituted to serine, was found to be viable at 30 °C but is completely inviable at 37 °C (8). Biochemical assays using purified WT and mutated Cab1p enzymes showed that the G351S substitution results in ∼94% loss of enzyme activity (9). Recent studies showed that the pantothenate analog α-PanAm² inhibits yeast growth in a pantothenic acid dose-dependent manner (9). The MIC₅₀ of the compound was found to be ∼4 times higher in medium supplemented with 1 μM pantothenic acid (MIC₅₀ ~ 7.6 μg/ml) compared with medium with 100 nM pantothenic acid (MIC₅₀ ~ 1.9 μg/ml) (9). In vitro pantothenate phosphorylation assays and MS analysis showed that α-PanAm is also phosphorylated by Cab1p and acts as a competitor of pantothenic acid at the enzyme catalytic

2 The abbreviations used are: α-PanAm, alpha-methyl-N-phenethyl-pantothenamide; MIC₅₀, minimum inhibitory concentration; ERG, ergosterol biosynthesis gene; ts, thermosensitive; T-MAStestis meiosis-activating sterol; PA, pantothenate; FIC, fractional inhibitory concentration; FPKM, fragments per kilobase of transcript per million mapped reads.

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Yeast pantothenate kinase regulates ergosterol biosynthesis

Reduced pantothenate phosphorylation results in altered yeast susceptibility to antifungals

Figure 1. Simplified CoA and ergosterol biosynthesis pathways in yeast. Shown is a schematic representation of key intermediates in the metabolic pathways for CoA and ergosterol biosynthesis in the yeast \textit{S. cerevisiae}. The CoA biosynthesis and ergosterol biosynthesis pathways are each boxed to demonstrate their separate treatment and lack of link in the current literature. The red arrow represents our novel conclusions, demonstrating the regulation that the CoA biosynthesis pathway exerts over the ergosterol biosynthesis pathway. Solid arrows represent single enzymatic steps between intermediates. Dashed arrows indicate multiple steps between intermediates. Further detailed enzymatic steps involved in these processes are shown in Fig. 2.
Biochemical analyses showed that the activity of the mutant $\text{Cab1}^{G351S}$ pantothenate kinase is only $\sim 7\%$ that of the WT at $30$ °C (Fig. 3B). The availability of the yeast $\text{cab1}^{ts}$ mutant strain made it possible to assess the effect of altered pantothenate kinase activity on yeast susceptibility to antifungals at $30$ °C. As shown in Fig. 3C, whereas the growth of the WT was inhibited by amorolfine, fluconazole, and terbinafine, the growth of the $\text{cab1}^{ts}$ mutant was not affected by these drugs. Conversely, with a sublethal dose of amphotericin B (1 μg/ml), the growth of the WT strain was only slightly inhibited, whereas that of the $\text{cab1}^{ts}$ mutant was reduced dramatically in the presence of the compound (Fig. 3C). MIC$_{50}$ values for these drugs against the two yeast strains were obtained for cell growth in liquid YPD medium (2% Bacto Peptone, 2% D-(+)-glucose, and 1% yeast extract). According to these values, the WT strain was about $10\times$ more susceptible to amorolfine, about $30\times$ more susceptible to fluconazole, and about $150\times$ more susceptible to terbinafine than the $\text{cab1}^{ts}$ strain. Conversely, $\text{cab1}^{ts}$ cells were about $5\times$ more susceptible to amorolfine than WT cells (Fig. 3D).

**Figure 2. Detailed metabolic pathway from pantothenate to ergosterol.** The pathway is divided into three subpathways: the CoA biosynthesis pathway from pantothenate (first box), the mevalonate pathway (second box), and the distal ergosterol biosynthetic pathway (third box). Solid arrows represent single enzymatic steps. Dashed arrows indicate multiple steps, catalyzed by the proteins in the order they are listed, from one intermediate to the next. Drug classes, individual drugs, and their site of action are shown in bold. Vit, vitamin; AAT, amino acid transporter; P-$\alpha$-PanAm, phosphorylated $\alpha$-PanAm.

**Inhibition of pantothenate utilization results in reduced susceptibility to terbinafine**

To further investigate the link between pantothenate utilization and antifungal susceptibility, we examined the effect of inhibition of Cab1p activity on yeast susceptibility to terbinafine. Consistent with its defect in pantothenic acid utilization, the growth of the $\text{cab1}^{ts}$ mutant under permissive conditions was ameliorated with pantothenic acid supplementation as the times to mid-log phase were 48, 36, and 28 h for cells grown in media supplemented with 1, 10, and 100 μM pantothenic acid, respectively (Fig. 4, B, D, and F). Comparatively, the growth of the WT strain was not significantly altered by increasing concentrations of pantothenic acid as the times to mid-log phase were $\sim 26$, 24, and 24 h at 1, 10, and 100 μM pantothenic acid, respectively (Fig. 4, A, C, and E). Interestingly, whereas the growth of the WT was dramatically decreased in media supplemented with $1 \mu$M pantothenic acid in the presence of terbinafine (relative time to mid-log phase of 1.8 in the presence versus absence of the drug), the growth of the $\text{cab1}^{ts}$ mutant was only moderately affected by the drug (relative time to mid-log phase of 1.1).
phase of 1.1 in the presence versus absence of the drug) (Fig. 4G). Addition of pantothenic acid to the culture medium at 10 or 100 μM resulted in higher sensitivity of both the WT (relative time to mid-log phase of 2.1 and 2.3 in the presence of 10 and 100 μM, respectively) and the cab1ts mutant (relative time to mid-log phase of 1.4 and 1.6 in the presence of 10 and 100 μM, respectively) to terbinafine (Fig. 4G).

Because reduced activity of Cab1p in cab1ts cells correlated with reduced susceptibility to terbinafine, we reasoned that chemical inhibition of Cab1p activity and/or CoA biosynthesis in the WT could also result in reduced susceptibility to the drug. We therefore examined the susceptibility of the WT to terbinafine in the presence of the pantothenamide α-PanAm (9). As shown in the isobologram in Fig. 4H, terbinafine and α-PanAm displayed a typical drug–drug antagonism pattern in the WT, suggesting that inhibition of pantothenate utilization and CoA biosynthesis results in reduced susceptibility to terbinafine.

\[ \text{Inhibition of pantothenate utilization results in enhanced susceptibility to amphotericin B} \]

Our studies also showed that the sensitivity of WT S. cerevisiae to amphotericin B depended on pantothenic acid availability. Whereas the growth rate of the WT strain was only moderately inhibited by 1 μg/ml amphotericin B in media supplemented with 100 μM pantothenic acid, it was significantly affected in media containing 100 μM pantothenic acid (relative time to mid-log phase of 2.1 in the presence versus absence of the drug) (Fig. 5, A, C, E, and G). Interestingly, the cab1ts mutant showed a much higher sensitivity to amphotericin B compared with the WT (Fig. 5, B, D, F, and G), indicating that reduced pantothenate kinase activity leads to increased sensitivity to this drug. Consistent with this phenotype, drug–drug interactions showed synergistic effects between amphotericin B and α-PanAm (Fig. 5H).

\[ \text{Inhibition of Cab1p activity results in reduced squalene and lanosterol levels} \]

To investigate the mechanism by which modulation of Cab1p activity alters yeast susceptibility to antifungals, we compared the levels of ergosterol, squalene, and other predominant sterol precursors by gas chromatography–mass spectrometry (GC–MS) between WT and cab1ts strains under permissive conditions. Ergosterol comprised the largest percentage of total sterols in both WT and cab1ts cells followed by the open-chain sterol precursor squalene. All other sterols each comprised no
more than ~10% of total sterols (Fig. 6A). As shown in Fig. 6B, reduced pantothenate phosphorylation in the cab1Δts mutant resulted in a significant reduction in the levels of squalene and lanosterol and a significant increase in ergosterol content. Consistent with these findings, supplementation of the growth medium of WT cells with 100 μg/ml squalene had only a slight effect on cell growth (mid-log phase reached at 28 h in the absence of squalene versus 34 h in the presence of squalene), whereas in the presence of terbinafine, the growth of WT cells was dramatically reduced (mid-log phase reached at 47 h in the absence of squalene versus 61 h in the presence of squalene) (Fig. 6, C and D).

To determine whether the reduced level of squalene seen in the cab1Δts mutant could also be due to changes in the transcription of the ERG1 gene or other genes in the ERG biosynthetic pathway, we performed RNA-Seq analysis on WT and cab1Δts strains grown under permissive conditions (minimal medium supplemented with 100 μM pantothenic acid). Two high-quality mRNA samples were selected for RNA-Seq analysis from two independent replicates, and the expression of the CoA biosynthesis genes (CAB1–5), ergosterol biosynthesis genes (20 ERG genes), and the transcriptional factors UPC2 and ECM22 was examined (Fig. 7A). Of the 20 ERG genes analyzed, the expression of ERG1, ERG11, ERG28, and ERG2 was significantly induced in the cab1Δts mutant compared with WT at 1.8-, 2.5-, 4.6-, and 2.4-fold, respectively (Fig. 7B). The ERG4 gene, a sterol reductase catalyzing the final step in ergosterol biosynthesis, was the only gene in the ERG biosynthesis pathway found to be down-regulated in the mutant, although not significantly. The expression of the transcriptional factors UPC2 and ECM22 was not significantly different between the WT and the cab1Δts mutant (Fig. 7B). As a control, no significant differences between the WT and cab1Δts strains could be detected in the expression levels of the housekeeping ACT1 gene (Fig. 7B).

Discussion

In this study, we provide the first evidence that changes in pantothenate phosphorylation, catalyzed by the pantothenate kinase Cab1p, control the metabolism of ergosterol and susceptibility of yeast cells to antifungal drugs. The CAB1 gene encodes the only pantothenate kinase in S. cerevisiae and is essential for cell viability. Other fungi, including all Candida
spp., A. fumigatus, Cryptococcus neoformans, and Histoplasma capsulatum, also express a single-copy gene encoding pantothenate kinase activity. Therefore, the encoded activity not only represents a promising new target in antifungal therapy but also a novel strategy to modulate the susceptibility of fungi to currently used antifungal drugs.

We took advantage of the availability of a thermosensitive cab1ts strain, which expresses mutated Cab1p enzyme with a substitution of glycine 351 to serine, to examine the possible link between pantothenate utilization and both ergosterol metabolism and yeast susceptibility to antifungals. This amino acid substitution results in a dramatic decrease (>90%) in enzyme catalysis sufficient to allow cells to survive at 30 °C but not at 37 °C. Cell growth assays showed that at 30 °C the cab1ts mutant is less susceptible to the antifungal drugs terbinafine, amorolfine, and fluconazole (all of which inhibit enzymes in late ergosterol biosynthesis) and more susceptible to amphotericin B (a drug that acts by forming 1:1 adducts with ergosterol rather than acting as an enzyme inhibitor) than the wild-type strain. The reduced sensitivity of the cab1ts mutant to terbinafine was reversed in media supplemented with high concentrations of pantothenic acid. These data suggest that changes in the utilization of pantothenic acid to CoA through inhibition or activation of the pantothenate kinase modulate yeast sensitivity to antifungals that target ergosterol biosynthesis. This novel control mechanism was further investigated by mimicking the reduced pantothenate utilization activity of the cab1ts strain by treating WT cells with the pantothenate analog α-PanAm (9). This compound was previously shown in both yeast cells and Plasmodium falciparum to inhibit CoA synthesis from pantothenic acid (9, 10). Consistent with the phenotype of the cab1ts mutant, treatment of WT cells with α-PanAm resulted in decreased susceptibility to terbinafine and increased susceptibility to amphotericin.

To investigate the direct impact of pantothenate utilization on ergosterol metabolism, we compared sterol and gene expression levels between WT and cab1ts strains. Of all sterols and precursors analyzed, squalene and ergosterol were the most predominant in both strains with significantly different levels detected in the cab1ts mutant compared with the WT (Fig. 6). All other detected sterols, including lanosterol, T-MAS, fecosterol, lichesterol, episterol, ergosta-5,7-dien-3β-ol, and

Figure 5. Relationship between pantothenate utilization and yeast susceptibility to amphotericin B. A–F, growth curves of WT and cab1ts cells without or with 1 μg/ml amphotericin B (amphB) in liquid minimal medium supplemented with varying concentrations of pantothenic acid. MM, minimal medium. G, relative times for treated WT and cab1ts cells to reach mid-log phase compared with untreated cells, calculated from A. Time for untreated cells to grow to mid-log was normalized to 1 and is represented by the dashed line. The asterisk (*) denotes that no relative times could be determined for the cab1ts strain because the treated cells did not grow and never reached mid-log phase. H, isobologram of the interaction between amphotericin B and the pantothenate analog α-PanAm (n = 2). The solid line represents the theoretical curve of an additive effect. Data below the solid line (dashed curve) show synergism between the two compounds.
Yeast pantothenate kinase regulates ergosterol biosynthesis

Figure 6. Effect of altered pantothenate phosphorylation on cellular sterol levels and sensitivity to terbinafine. A, the most prominent ergosterol precursors in the ergosterol biosynthetic pathway were chosen for analysis in both WT and cab1<sup>ts</sup> cells. B, levels of squalene, lanosterol, and ergosterol in WT cells were normalized to 1 and are represented by the dashed line on the graph. Bars denote the fold change in the relative level of each sterol in cab1<sup>ts</sup> cells compared with WT. *, p value 0.0088; **, p value 0.0185; ***, p value 0.0009. C, growth of WT cells in defined liquid media with 100 μM PA supplemented with squalene, terbinafine (terb), or both (n = 3). D, relative times of treated cells to grow to mid-log phase compared with untreated cells, calculated from values in Fig. 6C. Untreated cells were normalized to 1. *, p value 0.0017; **, p value 0.0002; ***, p value < 0.0001. Error bars represent SD.

Figure 7. Effect of altered pantothenate phosphorylation on CAB and ERG gene transcription. A, heat map (log<sub>2</sub> scale) showing changes in the expression levels of two transcription factors for the ergosterol biosynthesis pathway, CAB genes, and ERG genes between WT and cab1<sup>ts</sup> cells grown in minimal media supplemented with 100 μM pantothenic acid. Two independent replicates of the WT strain are represented by the left two columns, and two replicates of the cab1<sup>ts</sup> strain are represented by the right two columns. B, -fold change of gene expression levels (FPKM) between WT and cab1<sup>ts</sup> strains (n = 2). For each gene, the level in the WT was set to 1 to normalize the data. The bars show relative fold change of cab1<sup>ts</sup> cells to WT for each gene. The relative FPKM levels of the ACT1 gene are shown for control. *, p value 0.0011; **, p value 0.0013; ***, p value 0.0003; ****, p value < 0.0004. Error bars represent SD.
fungisterol, were present only in low quantities. Interestingly, the differences in squalene and ergosterol levels between the two strains were detected even though the cells were cultured in the presence of 100 μM pantothenic acid, which activates the metabolism of ergosterol and stimulates the growth of the cab1ts to levels similar to those found in the WT strain. Equally interestingly, transcriptional analysis revealed that the expression levels of several ERG genes were increased in the cab1ts mutant, including the ERG1 gene involved in squalene epoxidation. This suggests that the low steady-state levels of squalene in the mutant are due to both reduced metabolic input from the CoA biosynthesis pathway and increased squalene utilization by Erg1p, leading to reduced accumulation of this intermediate in the presence of terbinafine and consequently reduced sensitivity to the drug. This was further demonstrated in our studies as the susceptibility of WT cells to terbinafine was found to increase dramatically in the presence of excess squalene (Fig. 6C).

Our studies have shown that, in addition to its reduced susceptibility to the squalene epoxidase inhibitor terbinafine, the cab1ts mutant also displays reduced susceptibility to amorpholine and fluconazole. Whereas fluconazole targets the Erg11p enzyme (a cytochrome P450 enzyme), amorpholine targets both the Erg24p and Erg2p enzymes, catalyzing double bond modifications in the sterol backbone. Consistent with the reduced susceptibility of cab1ts cells to amorpholine, the levels of lanosterol, the substrate of Erg11p, were significantly lower in the cab1ts mutant compared with the WT, whereas the transcription of its encoding gene was increased. Additionally, we found that the transcription levels of ERG24 and ERG2 genes were significantly higher in the cab1ts mutant than in the WT. Together, the data are consistent with the model that a reduced input from the CoA pathway into the ergosterol biosynthesis pathway and a concomitant increase in the expression of the ERG genes involved in both the mevalonate and distal ergosterol biosynthesis pathways result in reduced levels of toxic intermediates of ergosterol biosynthesis, which would otherwise accumulate upon enzyme inhibition by drug classes targeting the distal synthesis pathway. Conversely, the increased susceptibility of the cab1ts strain to amphotericin B, which forms adducts with ergosterol on the membrane (9), was enhanced compared with the WT. Consistent with this finding, we analyzed the top 60 most-changed genes in global yeast transcription (Fig. S1). We found expression of DAN1 and DAN4, which are involved in the storage of ergosterol (15), to be significantly increased in the cab1ts mutant (Fig. S2A). Furthermore, expression of ARE1, ARE2, and NPC2 genes involved in the storage of sterols (16, 17), were also altered in the mutant (Fig. S2B). Significant up-regulation of ARE2 was seen in the mutant, suggesting increased sterol esterification and storage in the endoplasmic reticulum. Additionally, significant down-regulation of NPC2 was observed in cab1ts (Fig. S2B), a genetic modulation that is typically associated with the phenotype of massive lysosomal accumulation of sterols (17). These data suggest that perhaps the cab1ts mutant stores higher amounts of ergosterol in organelles, resulting in the higher ergosterol content observed in Fig. 6. The other major family of intracellular sterol-binding proteins, the OSH gene family, was also examined for changes in transcription. This family has previously been implicated in sterol transport between organelles (18).

Data showed that the family was not systemically up- or down-regulated, although the transcription of OSH5 was significantly reduced (Fig. S2C). Finally, transcription of genes associated with sterol export were studied, particularly the PRY gene family. Pry1p and Pry2p are secreted sterol-binding proteins, whereas Pry3p is cell wall–associated (19). PRY3 was significantly down-regulated in cab1ts, suggesting a possible decrease in the export of ergosterol in the mutant (Fig. S2D). In summary, available data suggest increased sterol uptake and storage and decreased sterol export in the cab1ts mutant, which may account for its increased susceptibility to amphotericin B. Consistent with our proposed model for amphotericin B sensitivity of the cab1ts mutant, studies by Hull et al. (20) showed that increased sterol uptake in Candida glabrata clinical isolates results in increased sensitivity to amphotericin B.

Altogether, our data suggest that pantothenate utilization controls ergosterol metabolism and the expression of ERG genes. Based on our model (Fig. 8), new strategies that exploit this mode of control by the pantothenate kinase may lead to better solutions for management of antifungal efficacy, toxicity, and resistance.

**Experimental procedures**

**Yeast strains**

*S. cerevisiae* strains used in this study were JS91.15–23 (WT: MATa his3 leu2 trp1 ura3) (8) and JS91.14–24 (cab1ts mutant: MATa ura3 his3 cab1ts) (8). WT and mutant strains were propagated either in YPD medium or defined pantothenic acid-free (minimal) medium composed of yeast nitrogen base (MP Biomedicals), supplemented with complete supplement mixture (MP Biomedicals) and all vitamins except pantothenic acid. Where indicated, media were supplemented with appropriate concentrations of pantothenic acid.

**Growth assays on solid and liquid media**

Spotting assays were performed as follows. Precultures of WT and cab1ts yeast strains were prepared in YPD medium overnight at 30 °C. Cells were harvested, washed, and diluted to 10⁸ cells in 500 μl of sterile water. Subsequent serial dilutions were made, and 5 μl of cell suspensions were spotted on YPD agar plates (square Petri dish with grid) lacking or supplemented with 10 ng/ml amorpholine, 1 μg/ml amphotericin B, 10 μg/ml fluconazole, or 10 μg/ml terbinafine to achieve 10⁶, 10⁵, 10⁴, 10³, 10², and 10¹ cells/spot. Plates were incubated at 30 or 37 °C and imaged every 24 h. For liquid assays in 96-well plate format, cells were precultured overnight in liquid YPD medium at 30 °C, washed three times in water, and diluted to achieve 10⁴ cells/well in 150 μl of minimal medium supplemented with 1, 10, or 100 μM pantothenic acid and either lacking or supplemented with terbinafine (160 μg/ml). All plates were incubated at 30 °C. Optical density measurements were taken with a BioTek SynergyMx microplate reader (OD₆₃₀) every 8 h for a total of 96 h. Relative time to mid-log phase was calculated by first graphing growth curves and then determining the time at which each curve reached mid-log growth saturation, chosen to be an OD of 0.6.
Pantothenate kinase activity assay

The activity of recombinant His₆-tagged Cab1p and Cab1Δ351p was determined in vitro using the Kinase-Glo Plus Luminescent Kinase (Kinase-Glo) assay as described previously (9). The assay measures the amount of ATP remaining in solution following the kinase reaction. Briefly, kinase buffer consisted of 100 mM Tris-HCl, pH 7.4, 10 mM MgCl₂, 100 μM pantothenate (PA), 100 μM ATP, and 0.5 mg/ml β-globulin. Kinase reactions were initiated with the addition of 500 ng of purified Cab1p or Cab1Δ351p in elution buffer (10 mM Tris, pH 7.4, NaCl 10 mM, and 500 mM imidazole). Heat-inactivated (20 min at 80 °C) Cab1p and Cab1Δ351p were used as controls. Samples were incubated at room temperature for 1 h. At the end of the incubation, an equal volume (10 μl) of Kinase-Glo reagent was added to each sample, the plates were further incubated for 5 min, and luminescence was recorded using a BioTek SynergyMx microplate reader (excitation wavelength, 485 nm; emission wavelength, 528 nm).

Drug–drug interactions and isobologram calculations

First, MIC₅₀ values for amorolfine, amphotericin B, fluconazole, and terbinafine against WT and cab1Δ cells grown in YPD liquid medium were calculated by the Clinical and Laboratory Standards Institute standard method M27-A3. Next, terbinafine–α-PanAm and amphotericin B–α-PanAm interactions were determined using liquid growth assays in 96-well plates using the checkerboard method as described previously (21). The isobologram was produced by solving for the sum 50% fractional inhibitory concentration (∑FIC₅₀) using the following equation where previously calculated MIC₅₀ values were used in the calculations.

\[
\sum \text{FIC}_5 = \frac{\text{MIC}_{50} \text{ of the drug combination}}{\text{MIC}_{50} \text{ of drug A alone}} + \frac{\text{MIC}_{50} \text{ of the drug combination}}{\text{MIC}_{50} \text{ of drug B alone}}
\]  

The ∑FIC₅₀ values calculated here were used to generate the isobologram curves.

Sterol analysis

Four independent 25-ml cultures of WT and cab1Δ were prepared in minimal medium supplemented with 100 μM PA at a starting cell density of 10⁴ cells/ml and incubated at 30 °C until they reached mid-log phase (average OD₆₃₀ ~0.6). Cells were then washed once in PBS, weighed, and stored at ~80 °C until used. The sterol pattern was determined by GC-MS as described previously (22, 23). The quantification, managed with an external calibration with ergosterol for the detected sterols and squalene for the content of squalene, consists of six levels with concentrations up to 10 g/ml. The base peak of each sterol trimethylsilyl ether (squalene and cholestane excluded) were taken as a quantifier ion for calculating the peak areas for squalene m/z 69, internal standard cholestane m/z 217, lichesterol (ergosta-5,8,22-trien-3β-ol) m/z 363, ergosterol (ergosta-5,7,22-trien-3β-ol) m/z 363, ergosta-5,7-dien-3β-ol m/z 365, fecosterol (ergosta-7,24(28)-dien-3β-ol) m/z 343, episterol (ergosta-7,24(28)-dien-3β-ol) m/z 343, fungisterol (ergost-7-en-3β-ol) m/z 472, lanosterol (4,4,14-trimethylcholesta-8,24-dien-3β-ol) m/z 393, and T-MAS (4,4-dimethylcholesta-8,24-dien-3β-ol) m/z 379.
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**Transcriptomic analysis**

RNA-Seq was performed to compare the transcriptional profiles of the WT and the cab1Δ strains grown in minimal medium with 100 μM pantothenic acid (conditions at which the strains showed equal growth). RNA was isolated from cultures of WT and cab1Δ strains using the RiboPure™RNA Purification kit (Invitrogen), and RNA-Seq was performed by running the samples on a HiSeq2500 in high-output mode 1 × 75 (200 million reads/lane). The reads were trimmed for quality and aligned with the hg19 reference genome using TopHat2 (24). The transcripts were assembled using Cufflinks (25). The assembled transcripts were used to estimate transcript abundance and differential gene expression using the Cuffdiff program (25). The results were visualized using R (CRAN) and CummeRbund (26).

**Statistics**

Relative times to mid-log phase of cab1Δ cells compared with WT cells in liquid media with terbinafine or amphotericin B treatment were analyzed by unpaired t tests with α value 0.05, resulting in two-tailed p values. For relative time to mid-log phase in 160 μg/ml terbinafine and 1 μM PA; p value <0.0001, t value = 23.52, df = 4, n = 3; 160 μg/ml terbinafine and 10 μM PA; p value <0.0001, t value = 43.43, df = 4, n = 3; and 160 μg/ml terbinafine and 100 μM PA; p value <0.0001, t value = 54.73, df = 4, n = 3. For relative time to mid-log phase in 1 μg/ml amphotericin B and 10 μM PA; p value = 0.8034, t value = 0.2386, df = 2, n = 3; 1 μg/ml amphotericin B and 100 μM PA; p value = 0.3505, t value = 1.208, df = 2, n = 3.

-Fold changes of the steroid squalene, lanosterol, and ergosterol were analyzed by paired t tests with α value 0.05, resulting in two-tailed p values. For -fold change squalene versus WT, p value = 0.0088, t value = 10.61, df = 2, n = 3. For -fold change lanosterol versus WT, p value = 0.0185, t value = 7.253, df = 2, n = 3. For -fold change ergosterol versus WT, p value = 0.0009, t value = 33.22, df = 2, n = 3.

Relative time of growth in liquid media for WT cells treated with squalene and/or terbinafine compared with untreated WT cells was analyzed by unpaired t tests with α value 0.05, resulting in two-tailed p values. For cells treated with 100 μg/ml squalene, 0 μg/ml terbinafine, p value = 0.0017, t value = 7.493, df = 4, n = 3. For cells treated with 0 μg/ml squalene, 80 μg/ml terbinafine, p value = 0.0002, t value = 12.64, df = 4, n = 3. For cells treated with 100 μg/ml squalene, 80 μg/ml terbinafine, p value <0.0001, t value = 52.56, df = 4, n = 3.

Comparison of RNA transcript levels in the form of FPKM values between WT and cab1Δ cells was statistically analyzed by unpaired t tests with α value 0.05, resulting in two-tailed p values. The following results were obtained: ACT1: p value = 0.1823, t value = 2.009, df = 2, n = 2; UPC2: p value = 0.9826, t value = 0.024, df = 2, n = 2; ECM22: p value = 0.4907, t value = 0.837, df = 2, n = 2; CAB1: p value = 0.2669, t value = 1.524, df = 2, n = 2; ERG1: p value = 0.0011, t value = 29.99, df = 2, n = 2; ERG11: p value = 0.0013, t value = 28.21, df = 2, n = 2; ERG2: p value = 0.1965, t value = 1.909, df = 2, n = 2; ERG28: p value = 0.0003, t value = 54.77, df = 2, n = 2; ERG2: p value = 0.0004, t value = 47.94, df = 2, n = 2; ERG4: p value = 0.1103, t value = 2.755, df = 2, n = 2; DAN1: p value = 0.0198, t value = 7.008, df = 2, n = 2; DAN4: p value = 0.0052, t value = 13.78, df = 2, n = 2; AUS1: p value = 0.3317, t value = 1.27, df = 2, n = 2; PDR11: p value = 0.2838, t value = 1.451, df = 2, n = 2; SUT1: p value = 0.8514, t value = 0.2125, df = 2, n = 2; ARE1: p value = 0.0266, t value = 6.014, df = 2, n = 2; ARE2: p value = 0.0189, t value = 7.17, df = 2, n = 2; NPC2: p value = 0.0482, t value = 4.388, df = 2, n = 2; YFT2: p value = 0.0609, t value = 3.866, df = 2, n = 2; OSH7: p value = 0.1107, t value = 2.75, df = 2, n = 2; OSH1/WH1: p value = 0.3304, t value = 1.275, df = 2, n = 2; OSH2: p value = 0.1786, t value = 2.037, df = 2, n = 2; OSH3: p value = 0.4847, t value = 0.8503, df = 2, n = 2; OSH4/KES1: p value = 0.0669, t value = 3.67, df = 2, n = 2; OSH5/HE1: p value = 0.0195, t value = 7.063, df = 2, n = 2; OSH6: p value = 0.6636, t value = 0.5053, df = 2, n = 2; PRY1: p value = 0.071, t value = 3.536, df = 2, n = 2; PRY2: p value = 0.0604, t value = 3.882, df = 2, n = 2; PRY3: p value = 0.0085, t value = 10.76, df = 2, n = 2.

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**References**


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