Candida glabrata drug:H\textsuperscript{+} antiporter CgTpo3 (ORF CAGL0I10384g): role in azole drug resistance and polyamine homeostasis

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Objectives: The ability of opportunistic pathogenic Candida species to persist and invade specific niches in the human host depends on their resistance to natural growth inhibitors and antifungal therapy. This work describes the role of the Candida glabrata drug:H\textsuperscript{+} antiporter CgTpo3 (ORF CAGL0I10384g) in this context.

Methods: Deletion and cloning of CgTPO3 was achieved using molecular biology tools. C. glabrata strain susceptibility was assayed based on growth in liquid and solid media and through MIC determination. Radiolabelled compound accumulation or HPLC were used for the assessment of the role of CgTpo3 as a drug or polyamine transporter. Quantitative RT–PCR was used for expression analysis.

Results: CgTpo3 was found to confer resistance to azole drugs in C. glabrata. This protein was found to be localized to the plasma membrane and to decrease the intracellular accumulation of \textsuperscript{3}H]clotrimazole, playing a direct role in its extrusion from pre-loaded C. glabrata cells. CgTPO3 was further found to confer resistance to spermine, complementing the susceptibility phenotypes exhibited by the deletion of its Saccharomyces cerevisiae homologue, TPO3. In spermine-stressed C. glabrata cells, CgTPO3 is transcriptionally activated in a CgPdr1-dependent manner, contributing to a decrease in the intracellular concentration of this polyamine. Clotrimazole exposure was found to lead to the intracellular accumulation of spermine, and pre-exposure to this polyamine was found consistently to lead to increased clotrimazole resistance.

Conclusions: Altogether, these results point to a significant role for CgTpo3 in azole drug resistance and in the tolerance to high polyamine concentrations, such as those found in the urogenital tract.

Keywords: azoles, multidrug resistance, drug:H\textsuperscript{+} antiporters, polyamines, CgPdr1

Introduction

Infections caused by Candida species, a problem of increasing clinical significance, are recognized as the 4th or 5th most common cause of nosocomial infections. Candida glabrata infections rank second in frequency after those caused by Candida albicans.\textsuperscript{1} The frequency and relatively high mortality levels (up to 45\% for C. glabrata) of these infections are generally attributed to the capacity of these pathogenic yeasts to efficiently develop multidrug resistance (MDR).\textsuperscript{5,6} In the particular case of C. glabrata, concern is further raised by the observation that clinical isolates are often resistant to azoles, the front-line drugs used in the treatment and prophylaxis against fungal pathogens.

Antifungal drug resistance in clinical isolates has been extensively linked to the activation of multidrug efflux pumps such as ATP-binding cassette (ABC) pumps and major facilitator superfamily (MFS) pumps, proposed to actively extrude or compartmentalize drugs and other xenobiotics, thus providing protection from these compounds.\textsuperscript{4,5} In C. glabrata, antifungal drug resistance has been shown to rely on the ABC drug efflux pump genes CgCDR1, CgPDH1/CgCDR2 and CgSNQ2.\textsuperscript{6,7} However, the role of the MDR transporters from the MFS, belonging to the drug:H\textsuperscript{+} antiporter (DHA) family, has received much less attention in the context of antifungal drug resistance, particularly concerning Candida species. In C. albicans, the DHA transporter Mdr1 was found to be linked to fluconazole resistance,\textsuperscript{6} a similar role being attributed...
to Mdr1 from Candida dubliniensis. In C. glabrata, recent work from our group demonstrated that the MFS-MDR transporters CgDqr2 and CgAqr1 are determinants of imidazole and flu- cytosine drug resistance, respectively. In C. glabrata, there are a total of 15 predicted DHA transporters, of which 10 belong to the DHA1 family, predicted to have 12 transmembrane spanners, and 5 to the DHA2 family, predicted to have 14 transmembrane segments.12 Interestingly, in the case of the model yeast Saccharomyces cerevisiae, most of the studied DHA transporters ended up being linked to MDR, despite the fact that their existence was only uncovered upon the release of the S. cerevisiae genome sequence. It is expected that most of these transporters in pathogenic yeasts and particularly in C. glabrata, which remain mostly uncharacterized, may play a significant role in MDR, with important implications in the development of intrinsic or acquired drug resistance in clinical isolates.

This article describes the functional analysis of the C. glabrata CgTPO3 gene, with emphasis on its role in antifungal drug resistance. This ORF encodes a putative DHA membrane transporter, found to confer resistance to inhibitory concentrations of compounds, and polyamines (apparently exhibiting some specificity towards spermine), and consistently to catalyse spermine excretion. In this study, the role and transcriptional regulation of the C. glabrata CgTPO3 gene in antifungal drug resistance was investigated. CgTpo3 was found to confer resistance to imidazole and triazole antifungal drugs. The subcellular localization of this transporter was assessed and its physiological role in CgTpo3 in polyamine homeostasis was evaluated and its activity in conferring MDR and regulation by the Cgdpr1 transcription factor investigated.

Materials and methods

Strains, plasmids and growth media

S. cerevisiae strain BY4741 (MATa, ura3Δ0, leu2Δ0, his3Δ1, met15Δ0) and the derived single deletion mutant BY4741Δtpo3 were obtained from the Euroscarf collection. The CBS138 C. glabrata strain, whose genome sequence was released in 2004, and KUE100 were used in this study. C. glabrata strain 5L51 (cgra3Δ0, cglu2Δ0) was kindly provided by John Bennett from the National Institute of Allergy and Infectious Diseases, NIH, Bethesda, USA. C. glabrata strains DSY562 (Δcgdpr1), SFY98 (DSY562 + Pdr1DSY86), SFY99 (DSY562 + Pdr1DSY86_GOF1328F), SFY114 (DSY562 + Pdr1DSYS65) and SFY115 (DSY562 + Pdr1DSYS65_GOF1328F) were kindly provided by Dominique Sanglard at the Institute of Microbiology, University of Lausanne and University Hospital Center, Lausanne, Switzerland. The plasmid pGREG576 was obtained from the Drag & Drop collection. Cells were batch cultured at 30°C with orbital agitation (250 rpm) in yeast extract peptone dextrose (YPD) growth medium with the following composition: 20 g of glucose (Merck), 20 g of yeast extract (Difco) and 10 g of peptone (Difco). For some of the experiments minimum medium was used, made by amino acid supplementation of basal medium (BM) with the following composition (per litre): 1.7 g of yeast nitrogen base without amino acids or NH4Cl, 20 g of glucose (Merck) and 2.65 g of (NH4)2SO4 (Merck). For some of the experiments minimal medium was used, consisting of BM supplemented with 20 mg of methionine, 20 mg of histidine, 60 mg of leucine and 20 mg of uracil (all from Sigma). C. glabrata strains derived from CBS138 and KUE100 or 5L51 were cultured in BM without supplementation or supplemented with 20 mg/L uracil and 60 mg/L leucine, respectively. To maintain selective pressure over the recombinant strains, the addition of uracil to this medium was only carried out to grow the host yeast cells. Agarized solid media contained, besides the previously indicated ingredients, 20 g/L agar (Iberagar).

Cloning of the C. glabrata CgTPO3 gene (ORF CAGL0I10384g)

The pGREG576 plasmid from the Drag & Drop collection was used to clone and express the C. glabrata ORF CAGL0I10384g in S. cerevisiae, as described previously for other heterologous genes. The pGREG576 was acquired from Euroscarf and contains a galactoside inducible promoter (GAL1), the yeast selectable marker URA3 and the GFP gene, encoding a green fluorescent protein (GFP565T), which allows monitoring of the expression of the gene in S. cerevisiae strain BY4741 with the pGREG576 vector, previously cut with the restriction enzyme SacI, to obtain the pGREG576_CgTPO3 plasmid. Since the GAL1 promoter only allows a slight expression of downstream genes in C. glabrata, a new construct was obtained to visualize by fluorescence microscopy the subcellular localization of the CgTPO3 gene in C. glabrata. The GAL1 promoter present in the pGREG576_CgTPO3 plasmid was replaced by the copper-induced Mti C. glabrata promoter, giving rise to the pGREG576_MTI_CgTPO3 plasmid. The Mti promoter DNA was generated by PCR, using genomic DNA extracted from the sequenced CBS138 C. glabrata strain, and the following specific primers: 3′-GAATTCGATATCAAGCTTATCGATACCGTCGACA-5′ and 5′-GAAAGTTCCTCCTACATGTCGAGCG-3′. The designed primers contain, besides a region with homology to the first and last 22 nucleotides of the CAGL0I10384g coding region (italic), nucleotide sequences with homology to the cloning site flanking regions of the pGREG576 vector (underlined). The amplified fragment was co-transformed into the parental S. cerevisiae strain BY4741 with the pGREG576 vector, previously cut with the restriction enzyme SacI, to obtain the pGREG576_CgTPO3 plasmid. The deletion of the C. glabrata CgTPO3 gene was carried out in the parental strain BY4741 with the pGREG576_CgTPO3 plasmid, previously cut with SacI and NotI restriction enzymes to remove the GAL1 promoter, to generate the pGREG576_MTI_CgTPO3 plasmid. The recombinant plasmids pGREG576_CgTPO3 and pGREG576_MTI_CgTPO3 were obtained through homologous recombination in S. cerevisiae and verified by DNA sequencing.

Disruption of the C. glabrata CgTPO3 gene (ORF CAGL0I10384g)

The deletion of the CgTPO3 gene was carried out in the parental strain KUE100 using the method described by Ueno et al. The target gene CAGL0I10384g (CgTPO3) was replaced by a DNA cassette including the CgHis3 gene, through homologous recombination. The replacement cassette was prepared by PCR using the following primers: 5′-AACACA GATAAAGGAAAAAGACCCCAAAAAGATTTACCCCAAAAATTAACAGGGG GCTGATACG-3′ and 5′-CAACTAAATTTCAATGCTGATCGTTA AAAACCC CTTTTTTTGTGTTGATACCCAGGTC-3′. The pHiSH906 plasmid including CgHis3 was used as a template and transformation was performed as described previously. Recombination locus and gene deletion.
were verified by PCR using the following pairs of primers: 5′-CAG CTTATCTCAGAACAACG-3′ and 5′-GACTTTGATGAGCGTGTAG-3′.

**Drug susceptibility assays**

The susceptibility testing of S. cerevisiae and C. glabrata strains against antifungal drugs or inhibitory polyamine concentrations was carried out in solid or liquid media or through standard MIC determination as described in the Supplementary Materials and methods (available at JAC Online).

**CgTpo3 subcellular localization assessment**

The subcellular localization of the CgTpo3 protein was determined based on the observation of BY4741 S. cerevisiae or LSU1 C. glabrata cells transformed with the pGREG576-CgTpo3 or pGREG576-MTI-CgTpo3 plasmids, respectively. These cells express the CgTpo3_GFP fusion protein, whose localization may be determined using fluorescence microscopy. S. cerevisiae cell suspensions were prepared by cultivation in MMU-U medium, containing 0.5% glucose and 0.1% galactose, at 30 °C with orbital shaking (250 rpm) until a standard culture with an optical density at 600 nm (OD<sub>600</sub>) of 0.4 ± 0.04 was reached. At this point, cells were transferred to the same medium containing 0.1% glucose and 1% galactose to induce protein expression. C. glabrata cell suspensions were prepared in BM until a standard culture with an OD<sub>600</sub> of 0.4 ± 0.04 was reached, and transferred to the same medium supplemented with 30 μM CuSO₄ (Sigma) to induce protein overexpression. After 5 h of incubation, the distribution of CgTpo2/3_GFP fusion protein in S. cerevisiae or C. glabrata living cells was detected by fluorescence microscopy using a Zeiss Axiosplan microscope (Carl Zeiss MicroImaging), using excitation and emission wavelengths of 395 and 509 nm, respectively. Fluorescence images were captured using a cooled, charge-coupled device camera (Cool SNAPFX, Roper Scientific Photometrics).

**[3H]clotrimazole transport assays**

[<sup>3</sup>H]clotrimazole transport assays were carried out as described previously. To estimate the internal accumulation of clotrimazole (intracellular/extracellular) inside yeast cells, the parental strain KUE100 and the mutant strain KUE100_Acgt3 were grown in BM to the mid-exponential phase and harvested by filtration. Cells were washed and resuspended in BM to obtain dense cell suspensions (OD<sub>600</sub> = 0.7 ± 0.1, equivalent to ~2.2 mg (dry weight)/mL). After 5 min incubation at 30 °C with agitation (150 rpm), 0.1 μM [<sup>3</sup>H]clotrimazole (American Radiolabelled Chemicals; 1 mL/cm²) and 30 mg/L unlabelled clotrimazole were added to the cell suspensions. Incubation proceeded for an additional period of 30 min. Additionally, for the [<sup>3</sup>H]clotrimazole efflux assays, yeast cells were resuspended in TM buffer (consisting of 0.1 M 2-(N-morpholino)ethanesulfonic acid (Sigma)/4 mM Tris (Sigma) adjusted to pH 4.5 with HCl) without glucose and incubated for 30 min at 30 °C with agitation (150 rpm) to de-energize the cell population. Then, 0.1 μM [<sup>3</sup>H]clotrimazole was added to the cell suspensions and the [<sup>3</sup>H]clotrimazole accumulation in de-energized yeast cells was followed until equilibrium was reached, after 30 min. Afterwards, a pulse of 2% glucose was added to the cell suspension and the efflux of [<sup>3</sup>H]clotrimazole was followed for an additional period of 30 min until a new equilibrium was reached. In all cases, the intracellular accumulation of labelled clotrimazole was followed by filtering 200 μL of cell suspension, at adequate time intervals, through pre-wetted glass microfiber filters (Whatman GF/F). The filters were washed with ice-cold TM buffer and the radioactivity measured using a Beckman LS 5000TD scintillation counter. Extracellular [<sup>3</sup>H]clotrimazole was estimated by radioactivity assessment of 50 μL of the supernatant.

Non-specific [<sup>3</sup>H]clotrimazole adsorption to the filters and to the cells (<5% of the total radioactivity) was assessed and taken into consideration to calculate the intracellular concentration of labelled clotrimazole, the internal cell volume of the exponential cells, grown in the absence of drug and used for accumulation assays, was considered constant and equal to 2.5 μL per mg dry weight. Statistical analysis of the results was performed using analysis of variance, and differences were considered statistically significant for P values <0.05.

**Intracellular spermine accumulation assessment**

This experiment was conducted based on the method described by Chan and Chua. Wild-type KUE100 and the Δcgt3 deletion mutant were cultured in liquid BM until a standardized culture with an OD<sub>600</sub> of 0.8 ± 0.08 was reached, then harvested and cultivated for 1 h in fresh media supplemented or not with 100 mg/L or 120 mg/L of clotrimazole. Alternatively, cells were cultured in liquid YPD medium until a standardized culture with an OD<sub>600</sub> of 0.8 ± 0.08 was reached, then harvested and cultivated for 1 h in fresh media supplemented or not with 4 mM spermine. To extract intracellular polyamines, these cells were resuspended in 1 mL of 10% trichloroacetic acid and incubated at 80 °C for 1 h to lyse the cells. Polyamines were then derivatized by the addition of 0.5 mL of 2 M NaOH and 5 μL of benzylic chloride for 40 min at 30 °C. The benzylation was stopped by adding 1 mL of saturated NaCl, and the derivatized polyamines were extracted with 1 mL ethyl ether and solubilized in methanol. Derivatized polyamine extracts were then collected and analysed with a 250 mm × 4 mm C18 column (LiChroCART Purospher STAR RP-18 end-capped 5 μm) at 40 °C. Polyamines were eluted based on the method described by Hwang et al. using 50% (v/v) methanol in water for 0.5 min, followed by a linear gradient from 50% (v/v) to 85% (v/v) methanol in water for 6.5 min at a flow rate of 0.6 mL/min. This was followed by an isocratic profile at 85% (v/v) methanol in water for 5 min at a flow rate of 0.6 mL/min. Finally, a decrease over 2 min to 50% (v/v) methanol in water at 0.6 mL/min. Peaks corresponding to polyamines were detected using absorption spectrophotometry at a wavelength of 254 nm. One-millilitre volumes of the polyamine standards (ranging from 50 nM to 200 μM spermine, spermidine and putrescine; Sigma) were also benzylated using 0.5 mL 2 M NaOH and 5 μL benzylic chloride, and extracted with ethyl ether. Under the conditions used, the retention times for putrescine, spermidine and spermine were 9.1, 11.4 and 12.6 min, respectively. The experiments were conducted in triplicate and the amounts of intracellular polyamines were expressed as μM/OD<sub>600</sub>.

**CgTPO3 and CgPDR1 expression measurements**

The levels of CgTPO3 and CgPDR1 transcripts in C. glabrata cells were assessed by quantitative real-time PCR. Total RNA samples were obtained from cell suspensions harvested under control conditions (mid-exponential phase cells in the absence of drugs) or upon 1 h of exposure to 30 μg/mL clotrimazole and 4 mM spermine. Synthesis of cDNA for real-time RT–PCR experiments, from total RNA samples, was performed using the Multiscribe™ Reverse Transcriptase Kit (Applied Biosystems) and a thermal cycler block (7500 Real-Time PCR System; Applied Biosystems), following the manufacturer's instructions. The quantity of cDNA for the following reactions was kept around 10 ng. The subsequent RT–PCR step was carried out using SYBR® Green reagents. Primers for the amplification of the CgTPO3, CgPDR1 and CgACT1 cDNA were designed using Primer Express Software (Applied Biosystems) and are 3′-TTGGCGTAGGTCATTGACG-5′ and 3′-TGGAGCGAAAGCGAAGAAAG-5′; 3′-CAGTTGCAACCCGTAGA-5′ and 3′-GACGACTTGGTAGGAGATCAT-5′ and 3′-AGACGCTTCTCCCTTCAT-5′ and 3′-TTGACCCATACGCAGCATGA-5′, respectively. RT–PCR was carried out using a thermal cycler block (7500 Real-Time PCR System; Applied Biosystems). Default parameters established by the manufacturer were used and fluorescence was detected by the instrument and registered in an amplification plot (7500 System SDS Software; Applied Biosystems). The CgACT1 mRNA level was used as an internal control. The relative values obtained for the wild-type strain...
under control conditions were set as 1 and the remaining values are presented relative to that control. To avoid false-positive signals, the absence of non-specific amplification with the chosen primers was confirmed by the generation of a dissociation curve for each pair of primers.

Results

*CgTPO3* expression confers resistance to azoles, similarly to *S. cerevisiae* TPO3

The deletion of the *CgTPO3* gene in *C. glabrata* was found, based on spot assays, to increase the susceptibility of this pathogen to the imidazole antifungal drugs tioconazole, miconazole, clotrimazole and ketoconazole; to the triazole fluconazole (Figure 1a); but not to the antifungals flucytosine or amphotericin B (results not shown). The MIC50 values of clotrimazole, miconazole, ketoconazole and fluconazole were also evaluated for these two strains using the standard CLSI (formerly NCCLS) method. MIC50 values were found to be in all cases higher for the parental KUE100 strain (2, 1, 0.25 and 32 mg/L, respectively) than for the Δcgtpo3 strain (1, 0.5, 0.125 and 8 mg/L, respectively), reinforcing the finding that *CgTPO3* appears to be a determinant of azole drug resistance in *C. glabrata*. The introduction of the single-copy pGREG576 plasmid expressing *CgTPO3* was found consistently to increase *C. glabrata* natural resistance towards clotrimazole, tioconazole and ketoconazole when compared with the same strain harbouring the corresponding cloning vector (Figure 1b).

Using *S. cerevisiae* as a heterologous expression system, the effect of *CgTPO3* expression on yeast resistance to antifungal drugs was further tested in order to verify whether or not *CgTPO3* is able to functionally complement its *S. cerevisiae* homologue. The deletion of the *S. cerevisiae* TPO3 gene was found to increase the susceptibility towards miconazole, tioconazole, miconazole, clotrimazole, fluconazole and itraconazole exhibited by the corresponding parental strain (Figure 1c). When expressed in the *S. cerevisiae* Δtpo3 background, the *CgTPO3* gene was able to rescue all the observed susceptibility phenotypes, further confirming its role in azole drug resistance (Figure 1c). No role in resistance to the polyene amphotericin B or to the fluoropyrimidine analogue flucytosine could be detected, either for the *S. cerevisiae* TPO3 gene, or for *C. glabrata* CgTPO3 (results not shown).

*CgTpo3* is localized to the plasma membrane in *C. glabrata* and when heterologously expressed in *S. cerevisiae*

*C. glabrata* cells harbouring the pGREG576_MTI_CgTPO3 plasmid were grown to mid-exponential phase in minimal medium and

![Figure 1](https://academic.oup.com/jac/article-abstract/69/7/1767/693817)

**Figure 1.** *CgTpo3* confers resistance to azole antifungal drugs in *C. glabrata* cells and functions as an orthologue of *ScTpo3* in *S. cerevisiae* cells. (a) Comparison of the susceptibility to antifungal azole drugs, at the indicated concentrations, of the *C. glabrata* KUE100 and KUE100_Δcgtpo3 strains on BM agar plates by spot assays. (b) Comparison of the susceptibility to antifungal azole drugs, at the indicated concentrations, of the *C. glabrata* L5U1 strain, harbouring the pGREG576 cloning vector (v) or pGREG576_CgTPO3, on BM agar plates, without uracil, by spot assays. (c) Comparison of the susceptibility to antifungal azole drugs, at the indicated concentrations, of the *S. cerevisiae* BY4741 and BY4741_Δtpo3 strains, harbouring the cloning vector pGREG576 (v) or the derived *CgTPO3* expression plasmid pREG_CgTPO3, on MM4 agar plates by spot assays. The inocula were prepared as described in the Materials and methods section. Cell suspensions used to prepare the spots were 1:5 (b) and 1:25 (c) dilutions of the cell suspensions used in (a). The displayed images are representative of at least three independent experiments.
then transferred to the same medium containing 30 μM CuSO₄ to induce the expression of the fusion protein. At a standard OD₆₀₀ of 0.5 ± 0.05, obtained after ~5 h of incubation, cells were inspected using fluorescence microscopy. In C. glabrata cells, the CgTpo3-GFP fusion was found to be predominantly localized to the cell periphery (Figure 2a). By contrast, control cells harbouring the pGREG576 cloning vector displayed a slight and uniform distribution of fluorescence (Figure 2a), similar to that observed as the host cells’ auto-fluorescence. The construction of a hydrophathy profile for the CgTpo3 amino acid sequence using TMHMM software (http://www.cbs.dtu.dk/services/TMHMM/), predicts its topology to include 12 transmembrane-α helical segments, with a cytoplasmic loop in between the six plus six transmembrane segment configuration (Figure S1, available as Supplementary data at JAC Online), as foreseen for the remaining members of the DHA1 family. 5,12 Altogether, these results strongly suggest a plasma membrane localization, similar to that observed for its S. cerevisiae homologue Tpo3. 14 S. cerevisiae cells harbouring the pGREG576_CgTpo3 plasmid were also tested for the subcellular localization of CgTpo3 to verify that in these cells the C. glabrata transporter was similarly localized to the plasma membrane. Cells were grown to mid-exponential phase in minimal medium containing 0.5% glucose and 0.1% galactose, and then transferred to the same medium containing 0.1% glucose and 1% galactose to promote protein overexpression. At a standard OD₆₀₀ of 0.5 ± 0.05, obtained after ~5 h of incubation, cells were inspected using fluorescence microscopy and plasma membrane localization was verified (Figure 2b).

**CgTpo3 mediates [³H]clotrimazole efflux in C. glabrata**

Since the C. glabrata CgTpo3 was identified as a plasma membrane MDR transporter conferring resistance to azole drugs, its possible involvement in reducing their accumulation in challenged yeast cells was examined, focusing on the antifungal drug clotrimazole. The accumulation of [³H]labelled clotrimazole in non-adapted C. glabrata cells suddenly exposed to the presence of 75 mg/L cold clotrimazole was assessed. Under these conditions, the deletion of the CgTPO3 gene increased drastically the duration of the clotrimazole-induced lag phase when compared with the parental strain. The observed lag phase corresponds to a period of mild cell viability loss, followed by prolonged growth arrest, prior to exponential growth resumption (Figure 3a). In cells devoid of CgTPO3, the intracellular accumulation of [³H]clotrimazole was found to be 2-fold higher than in parental KUE100 cells (Figure 3b). This result strongly suggests that CgTpo3 activity increases yeast resistance to clotrimazole by reducing its accumulation within yeast cells, presumably by catalysing the direct extrusion of this antifungal drug. To assess this possibility, the same cells were de-energized and, in the absence of glucose, exposed to [³H]-labelled clotrimazole. The passive accumulation of this radiolabelled antifungal drug reached similar levels in both KUE100 and KUE100 Δcgtpo3 cells (Figure S2, available as Supplementary data at JAC Online). Upon a glucose pulse, to drive energy-dependent transport mechanisms, [³H]clotrimazole was found to be extruded from these pre-loaded C. glabrata cells (Figure 3c). However, drug efflux was found to be much more efficient in wild-type KUE100 cells expressing CgTPO3 than in the derived Δcgtpo3 deletion mutant (Figure 3c). Altogether, these results suggest that CgTPO3 plays a direct role in catalysing the active export of clotrimazole from C. glabrata cells.

**CgTPO3 expression confers resistance to inhibitory concentrations of polyamines, similarly to S. cerevisiae TPO3**

Exploring the concept that DHA transporters may have a physiological role other than broad chemoprotection, and given the knowledge gathered for the S. cerevisiae TPO3 gene, 13,16 a possible role for CgTpo3 in the resistance to inhibitory concentrations of polyamines was investigated. Based on spot assays, the deletion of the CgTPO3 gene in C. glabrata was found to increase the susceptibility of this pathogen to the polyamines spermine, spermidine and putrescine (Figure 4a). The introduction of the pGREG576 single-copy plasmid expressing CgTPO3 increased C. glabrata natural resistance towards inhibitory concentrations of all polyamines, but particularly to spermine, when compared with the same strain harbouring the corresponding cloning vector (Figure 4b), reinforcing the finding that CgTPO3 is a determinant of polyamine resistance in C. glabrata. Using S. cerevisiae as a heterologous expression system, the effect of CgTPO3 expression in yeast resistance to polyamines was further tested in order to verify whether or not CgTPO3 is able to functionally complement its S. cerevisiae homologue. When expressed in the S. cerevisiae Δtpo3 background, the CgTPO3 gene was able to rescue its susceptibility to polyamines (Figure 4c).

**CgTPO3 plays a role in spermine homeostasis, but spermine accumulation in response to clotrimazole is independent of CgTPO3**

Based on the obtained results, the effect of CgTPO3 expression on the intracellular concentration of spermine was assessed. Under control conditions, no significant difference could be observed. Remarkably, when wild-type C. glabrata cells were exposed to inhibitory concentrations of clotrimazole, the intracellular concentration of spermine was found to increase around 20-fold.
However, this change was found to occur independently of CgTpo3 expression (Figure 5a). Finally, upon exposure to inhibitory concentrations of 4 mM spermine, there was a 7-fold accumulation of this polyamine inside C. glabrata cells. Consistent with the hypothesis that CgTpo3 might be involved in spermine excretion, this increase was found to be twice as much in the Dcgtpo3 deletion mutant (Figure 5b).

Given the accumulation of spermine registered in response to clotrimazole, we hypothesized that exposure to spermine might provide cross-protection against clotrimazole. To assess this possibility, the effect of pre-exposure or co-exposure to spermine in clotrimazole resistance was evaluated. CgTpo3 expression was observed to remain unaltered following 1 h of exposure of a non-adapted C. glabrata population to an inhibitory concentration of clotrimazole (Figure 6a). However, the transcript levels of the CgTpo3 gene were seen to increase 2-fold following 1 h of cultivation in the presence of an inhibitory concentration of spermine. Interestingly, under both control conditions and spermine-induced stress, the expression of CgTpo3 was found to be controlled by the CgPdr1 transcription factor (Figure 6a), selected for its role in the regulation of azole drug resistance development. 6 The transcript levels of CgPdr1 itself were found to be 1.5-fold increased in cells exposed to 4 mM spermine (Figure 6b). Given the observation that CgPdr1 is required for CgTpo3 up-regulation in spermine-challenged cells, its participation in spermine tolerance was further analysed through spot assays. Significantly, the deletion of CgPdr1 was different growth media, owing to the poor solubility of spermine in the BM.

**CgTPO3 transcript levels are up-regulated under spermine stress and are dependent on CgPdr1**

The effect on CgTpo3 transcription of C. glabrata cell exposure to the chemical stressors to which CgTpo3 confers resistance was evaluated. CgTpo3 expression was observed to remain unaltered following 1 h of exposure of a non-adapted C. glabrata population to an inhibitory concentration of clotrimazole (Figure 6a). However, the transcript levels of the CgTpo3 gene were seen to increase 2-fold following 1 h of cultivation in the presence of an inhibitory concentration of spermine. Interestingly, under both control conditions and spermine-induced stress, the expression of CgTpo3 was found to be controlled by the CgPdr1 transcription factor (Figure 6a). The transcript levels of CgPdr1 itself were found to be 1.5-fold increased in cells exposed to 4 mM spermine (Figure 6b). Given the observation that CgPdr1 is required for CgTpo3 up-regulation in spermine-challenged cells, its participation in spermine tolerance was further analysed through spot assays. Significantly, the deletion of CgPdr1 was
found to slightly increase \textit{C. glabrata} susceptibility to spermine-induced stress, whereas the expression of two gain-of-function (GOF) mutated forms of Pdr1 (L280F and L328F) were found to increase \textit{C. glabrata} resistance towards this stress (Figure S4, available as Supplementary data at \textit{JAC} Online).

**Discussion**

In this study, the functional characterization of the \textit{C. glabrata} CgTpo3 DHA was carried out. CgTpo3 was found to be the third in a family of 10 members to be associated with azole antifungal drug resistance in \textit{C. glabrata}. A physiological role is proposed for Tpo3 in the control of spermine intracellular concentration, and a link between the action of CgTpo3 as an MDR transporter and its physiological role as an eventual transporter of polyamines was evaluated.

Azole antifungal drugs to which CgTpo3 confers resistance include the imidazoles miconazole, ketoconazole, and clotrimazole, used in the treatment of fungal skin infections, including vaginal or oral candidaemia, and also triazoles, such as fluconazole and itraconazole, applied to control systemic fungal infections. In \textit{C. glabrata}, resistance to fluconazole among clinical isolates has been shown to depend often on the action of the ABC drug efflux pumps encoded by \textit{CgCDR1} and \textit{CgCDR2}. Furthermore, the ABC drug efflux pump encoded by \textit{CgAUS1} appears to function as a sterol transporter that may contribute to lower azole susceptibility in the presence of serum and to protect \textit{C. glabrata} against azole toxicity in vivo.\footnote{26} The eventual participation of the DHA family members in imidazole resistance has been shown very recently to include in \textit{C. glabrata} the action of the \textit{CgQDR2} gene.\footnote{13} However, in this study, CgTpo3 was identified as the first of its family to confer fluconazole resistance on \textit{C. glabrata}. Significantly, CgTpo3 has close homologues in other pathogenic \textit{Candida} species, which may also play a role in azole drug resistance in these related pathogenic yeasts, including those encoded by \textit{orf19.4737}, in \textit{C. albicans}, \textit{CPAR2_804310}, in \textit{Candida parapsilosis}, and \textit{Cd36_08290}, from \textit{C. dubliniensis}.

Although many of the characterized drug efflux pumps have been shown to confer resistance to a wide variety of drugs and chemicals,\footnote{27,28} the molecular mechanisms behind their apparent promiscuity remains elusive.\footnote{4,28–31} Based on its high degree of homology and functional similarity to the \textit{S. cerevisiae} TPO3 gene, a possible physiological role linked to yeast survival in the presence of inhibitory polyamine concentrations\footnote{15} was investigated. Indeed, CgTpo3 expression did improve \textit{C. glabrata} and \textit{S. cerevisiae} fitness under inhibitory spermine and putrescine concentrations, and complemented the susceptibility phenotype exhibited by the \textit{S. cerevisiae} \textit{Δtpo3} deletion mutant. Furthermore, \textit{C. glabrata} cells exposed to inhibitory concentrations of polyamines were found to accumulate this metabolite intracellularly. This accumulation was exacerbated in the \textit{Δcgtpo3} cells,

**Figure 4.** CgTpo3 confers resistance to azole antifungal drugs in \textit{C. glabrata} and \textit{S. cerevisiae} cells. (a) Comparison of the susceptibility to inhibitory concentrations of polyamines of the \textit{C. glabrata} KUE100 and KUE100_Δcgtpo3 strains on BM agar plates by spot assays. (b) Comparison of the susceptibility to inhibitory concentrations of polyamines of the \textit{C. glabrata} L5U1 strain, harbouring the pGREG576 cloning vector (v) or pGREG576_CgTPO3, on BM agar plates, without uracil, by spot assays. (c) Comparison of the susceptibility to inhibitory concentrations of polyamines of the \textit{S. cerevisiae} BY4741 and BY4741_Δtpo3 strains, harbouring the cloning vector pGREG576 (v) or the derived CgTPO3 expression plasmid pGREG_CgTPO3, on MM4 agar plates by spot assays. The inocula were prepared as described in the Materials and methods section. Cell suspensions used to prepare the spots were 1:5 (b) and 1:25 (c) dilutions of the cell suspensions used in (a). The displayed images are representative of at least three independent experiments.
consistent with a role for this transporter in spermine excretion. The possibility that the physiological role of CgTpo3 in polyamine homeostasis may contribute indirectly to its action as an MDR determinant was further investigated, given that this seems to be the case for the majority of the ABC and MFS-MDR transporters characterized in S. cerevisiae.4,5,32

The effect of clotrimazole on the intracellular concentration of spermine was examined and, interestingly, the concentration of spermine was found to increase dramatically in clotrimazole-challenged cells. This effect could not be attributed to the action of CgTpo3, suggesting that the role of this transporter in clotrimazole resistance is entirely due to its action on clotrimazole extrusion. The effect of clotrimazole on the intracellular concentration of spermine is per se a very interesting observation in terms of the mode of action of imidazole antifungals. Strong evidence has accumulated for a role of polyamines in microbial pathogenesis. In particular, it was demonstrated in Shigella species that intracellular accumulation of spermidine increases oxidative stress tolerance, allowing this pathogen to better survive in the host, particularly under the adverse conditions found inside macrophages.13 In C. albicans, the assimilation of polyamines as a carbon source, followed by the export of the amine group in the form of ammonia, leads to an increase in extracellular pH, which in turn results in the yeast-to-hyphae transition.16 Interestingly, C. glabrata, although unable to engage hyphal growth and thus escape macrophages, exhibits a remarkable ability to persist and survive inside the human host. Furthermore, C. glabrata displays a higher intrinsic resistance to oxidative stress than S. cerevisiae or C. albicans, which appears to correlate with its ability to persist in the host upon adhesion-induced extracellular host-derived reactive oxygen species (ROS).15 Altogether, the observed increase in the intracellular concentration of spermine upon clotrimazole challenge may indeed confer an advantage to C. glabrata cells. In agreement with this hypothesis, it was possible to verify that preexposure to spermine significantly increased C. glabrata resistance to an inhibitory concentration of clotrimazole. Although the exact rationale for the effect of clotrimazole in the intracellular spermine concentration was not clarified, it may further relate to the observation that imidazoles such as ketoconazole and miconazole,36 and most likely clotrimazole, induce an increase in plasma membrane polarization, apparently increasing K+ efflux and H+ influx in challenged cells. Given that polyamines such as spermine are polycations, the supplementation of the growth medium with inhibitory concentrations of polyamines is known to also induce membrane polarization. This fact may explain the observation that simultaneous exposure to clotrimazole and spermine has a dramatic growth inhibitory effect in C. glabrata cells. But if the concentration of polyamines increases intracellularly, it may serve precisely the goal of balancing the membrane hyperpolarization induced by clotrimazole, promoting the return of this key parameter to normal physiological levels. This interconnection between polyamine homeostasis andazole drug resistance may be particularly interesting in cases of Candida infection that take place in the human urogenital tract, where polyamine concentrations can reach 15 mM for spermine, 5 mM for spermidine and 3 mM for putrescine.37 The observed role for CgTpo3 in polyamine resistance may further be important to enable colonization and pathogenicity in this otherwise adverse infection niche.

Finally, the finding that CgPdr1 plays a role in the control of GTP03 gene expression suggests a link between this drug efflux pump and the major controller of the MDR phenomenon in C. glabrata. The obtained results are consistent with a previous
observation, based on microarray analysis, that in the absence of CgPdr1, the expression of CgTPO3 is down-regulated. An analysis of the CgTPO3 promoter region, performed in the Regulatory Sequence Analysis Tools web site, failed to identify any of the canonical CgPdr1 element (PDRE: BCCRRYRGD). However, if a single nucleotide substitution in the PDRE sequence is allowed, four putative CgPdr1 loci are found within the first 50–600 nucleotides upstream of the CgTPO3 coding sequence (Figure S5, available as Supplementary data at JAC Online). Although it remains to be determined whether the effect of CgPdr1 in CgTPO3 expression is direct or indirect, this link suggests that the action of this transporter, as described in this article, may also have a relevant impact in the clinical context. Furthermore, this observation also links CgPdr1 with polyamine stress resistance, which may be relevant in the context of urogenital Candida infections. Interestingly, the expression of CgPdr1, and particularly CgPdr1 GOF mutants, was found to confer resistance to inhibitory concentrations of spermine.

Altogether, the results described herein reinforce the idea that multidrug transporters from the MFS contribute to the overall resistance phenotype. Indeed, this study, characterizing the C. glabrata Tpo3 multidrug transporter involved in azole drug resistance and polyamine homeostasis, highlights the importance of studying the remaining members of this family in C. glabrata in this context, with an expected impact on the treatment of the increasing number of azole-resistant fungal infections.

**Figure 6.** CgTPO3 and CgPDR1 transcript levels are up-regulated under spermine-induced stress. (a) Comparison of the variation of the CgTPO3 transcript levels in wild-type (filled bars) or Δcgpdr1 (open bars) C. glabrata cells before (control) and after 1 h of exposure to clotrimazole- or spermine-induced stress, at the indicated concentrations. (b) Comparison of the variation in CgPDR1 transcript levels in wild-type C. glabrata cells before (control) and after 1 h of exposure to spermine-induced stress, at the indicated concentration. The presented transcript levels were obtained by quantitative RT–PCR, as described in the Materials and methods section, and are relative CgTPO3/CgACT1 or CgPDR1/CgACT1 mRNA values, considering the value registered under control conditions to be equal to 1. The obtained values are the average of at least three independent experiments. Error bars represent the corresponding standard deviations. *P<0.05. **P<0.01.

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### Transparency declarations

None to declare.

### Supplementary data

Supplementary Materials and methods and Figures S1 to S5 are available as Supplementary data at JAC Online (http://jac.oxfordjournals.org/).

### References


