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Candida glabrata drug:H^+ 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^+ 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 [^{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^+ 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*.[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).[2,3] 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.[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*.[6,7] However, the role of the MDR transporters from the MFS, belonging to the drug:H^+ 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,[8] a similar role being attributed

to Mdr1 from *Candida dubliniensis*.[9] In *C. glabrata*, recent work from our group demonstrated that the MFS-MDR transporters CgQdr2[10] and CgAqr1[11] are determinants of imidazole and flucytosine 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.[5] 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* ORF *CAGL0I10384g* (*CgTPO3* gene), with emphasis on its role in antifungal drug resistance. This ORF encodes a putative DHA from the MFS, sharing a high degree of homology with the *S. cerevisiae TPO3* gene.[13–15] *S. cerevisiae* Tpo3 is a plasma membrane transporter,[14] found to confer resistance to inhibitory concentrations of weak organic acids[15] and polyamines (apparently exhibiting some specificity towards spermine), and consistently to catalyse spermine excretion.[13,14] 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 action in the extrusion of [^{3}H]clotrimazole from pre-loaded *C. glabrata* cells was verified. Finally, a possible link between the physiological role of CgTpo3 in polyamine homeostasis was evaluated and its activity in conferring MDR and regulation by the CgPdr1 transcription factor investigated.

Materials and methods

Strains, plasmids and growth media

S. cerevisiae strain BY4741 (*MAT*a, ura3Δ0, leu2Δ0, his3Δ1, met15Δ0) and the derived single deletion mutant BY4741_*Δtpo3* were obtained from the Euroscarf collection. The CBS138 *C. glabrata* strain, whose genome sequence was released in 2004, and KUE100[16] were used in this study. *C. glabrata* strain L5U1 (cgura3Δ0, cgleu2Δ0) was kindly provided by John Bennett[17] from the National Institute of Allergy and Infectious Diseases, NIH, Bethesda, USA. *C. glabrata* strains DSY562 (*Δcgpdr1*), SFY98 (DSY562+Pdr1$_{DSY486}$), SFY99 (DSY562+Pdr1$_{DSY486}$_GOF$_{L328F}$), SFY114 (DSY562+Pdr1$_{DSY565}$) and SFY115 (DSY562+Pdr1$_{DSY565}$_GOF$_{L328F}$)[18] 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.[19]

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 minimal 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 NH_4^+ (Difco), 20 g of glucose (Merck) and 2.65 g of $(NH_4)_2SO_4$ (Merck). *S. cerevisiae* wild-type and derived BY4741 strains were grown in MM4 medium, 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 L5U1 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[19] was used to clone and express the *C. glabrata* ORF *CAGL0I10384g* in *S. cerevisiae*, as described previously for other heterologous genes.[20] pGREG576 was acquired from Euroscarf and contains a galactose inducible promoter (*GAL1*), the yeast selectable marker *URA3* and the *GFP* gene, encoding a green fluorescent protein (GFPS65T), which allows monitoring of the expression and subcellular localization of the cloned fusion protein. *CAGL0I10384g* DNA was generated by PCR using genomic DNA extracted from the sequenced CBS138 *C. glabrata* strain, and the following specific primers: 3′-GAATTCGATATCAAGCTTATCGATACCGTCGACA*ATGGTGGATCAAG AATCATTGG*-5′ and 3′-GCGTGACATAACTAATTACATGACTCGAGGTCGAC*TTA GTATCCTTCAATGGTATCG*-5′. 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 SalI, 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′-TTAACCCTCACTAAAGG GAACAAAAGCTGGAGCTC*TGTACGACACGCATCATGTGGCAATC*-5′ and 3′-GAA AAGTTCTTCTCCTTTACTCATACTAGTGCGGC*TGTGTTTGTTTTTGTATGTGTTTGT TG*-5′. The designed primers contain, besides a region with homology to the first and last 19 nucleotides of the first 1000 bp of the *MTI* promoter 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 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.*[21] 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 GATAAAAAGGAAAAAGACCCCCAACAGATAATCTTAACAAAAAATTACAAGGCC GCTGATCACG-3′ and 5′-CAATCAAATTTTCAAATGCTGCTTTGTTAAAAAACC CTTTTTTTATTTGTTGATTACATCGTGAGGCTGG-3′. The pHIS906 plasmid including *CgHIS3* was used as a template and transformation was performed as described previously.[16] Recombination locus and gene deletion

were verified by PCR using the following pairs of primers: 5′-CAG CTTTATCTCAGAAAACCAG-3′ and 5′-GACTTTGGATGACGCTGTAG-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 L5U1 *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 MM4-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_{600}) 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_{600} of 0.4 ± 0.04 was reached, and transferred to the same medium supplemented with 30 μM $CuSO_4$ (Sigma) to induce protein overexpression. After 5 h of incubation, the distribution of CgTpo2/3_GFP fusion protein in *S. cerevisiae* or in *C. glabrata* living cells was detected by fluorescence microscopy using a Zeiss Axioplan 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).

[^{3}H]clotrimazole transport assays

[^{3}H]clotrimazole transport assays were carried out as described previously.[10] To estimate the internal accumulation of clotrimazole (intracellular/extracellular) inside yeast cells, the parental strain KUE100 and the mutant strain KUE100_*Δcgtpo3* 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 [$OD_{600} = 0.7 \pm 0.1$, equivalent to ∼2.2 mg (dry weight)/mL]. After 5 min incubation at 30°C with agitation (150 rpm), 0.1 μM [^{3}H]clotrimazole (American Radiolabelled Chemicals; 1 mCi/mL) and 30 mg/L unlabelled clotrimazole were added to the cell suspensions. Incubation proceeded for an additional period of 30 min. Additionally, for the [^{3}H]clotrimazole efflux assays, yeast cells were resuspended in TM buffer [consisting of 0.1 M 2-(*N*-morpholino)ethanesulfonic acid (Sigma)/41 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 [^{3}H]clotrimazole was added to the cell suspensions and the [^{3}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 [^{3}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 prewetted glass microfibre filters (Whatman GF/C). The filters were washed with ice-cold TM buffer and the radioactivity measured using a Beckman LS 5000TD scintillation counter. Extracellular [^{3}H]clotrimazole was estimated by radioactivity assessment of 50 μL of the supernatant.

Non-specific [^{3}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.[22] 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.[23] Wild-type KUE100 and the Δ*cgtpo3* deletion mutant were cultured in liquid BM until a standardized culture with an OD_{600} 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_{600} 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 benzoyl chloride for 40 min at 30°C. The benzoylation 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 endcapped 5 μm) at 40°C. Polyamines were eluted based on the method described by Hwang *et al.*[24] 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 benzoylated using 0.5 mL 2 M NaOH and 5 μL benzoyl 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_{600}.

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 mg/L 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′-TGCCGATATGTTCCCAAGTGA-5′ and 3′-TGGAGCGAAAGCGAAGAAAG-5′, 3′-CGATTGCCAACCCGTTAGA-5′ and 3′-GACGACCTTGGTGTAGGAGTCAT-5′ and 3′-AGAGCCGTCTTCCCTTCCAT-5′ and 3′-TTGACCCATACCGACCATGA-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 MIC_{50} values of clotrimazole, miconazole, ketoconazole and fluconazole were also evaluated for these two strains using the standard CLSI (formerly NCCLS) method. MIC_{50} 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

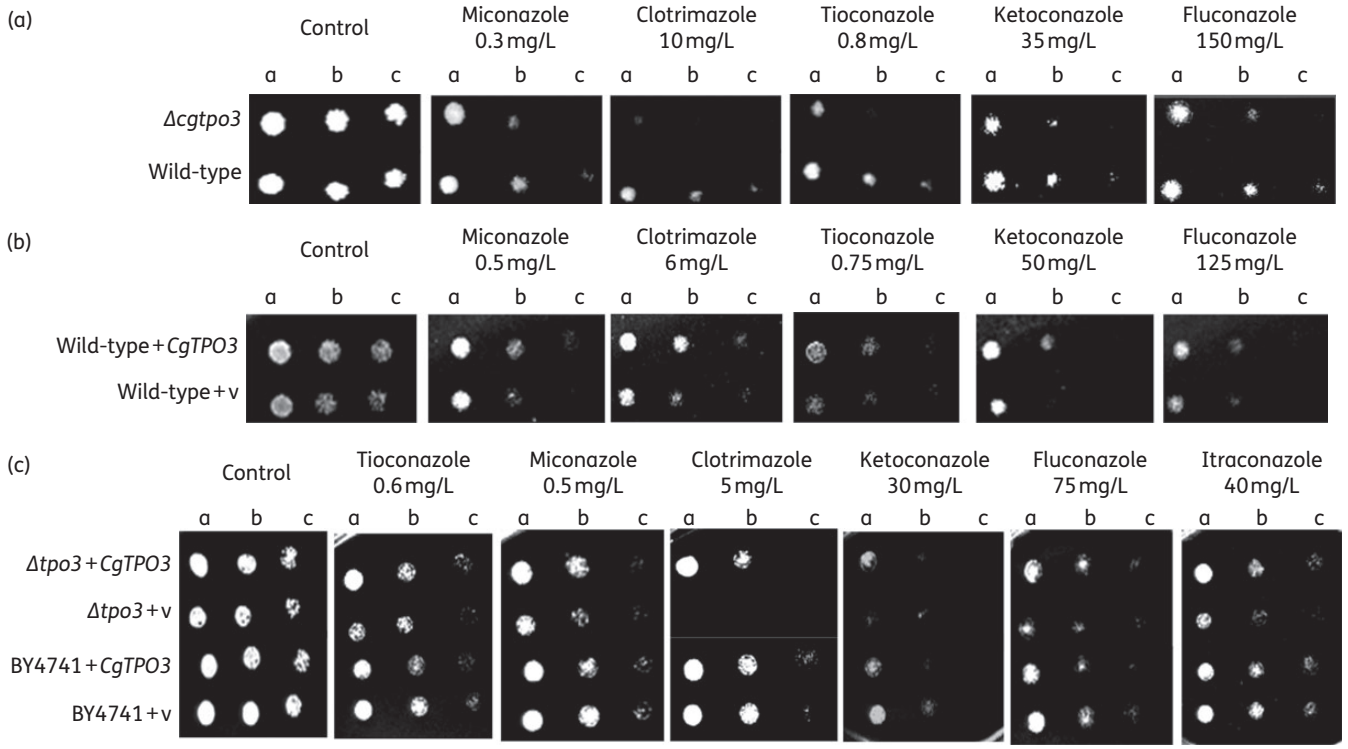

Figure 1. CgTpo3 confers resistance to azole antifungal drugs in *C. glabrata* cells and functions as an orthologue of ScTpo3 in *S. cerevisiae* cells. (a) Comparison of the susceptibility to antifungal azole drugs, at the indicated concentrations, of the *C. glabrata* KUE100 and KUE100_*Δcgtpo3* strains 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 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.

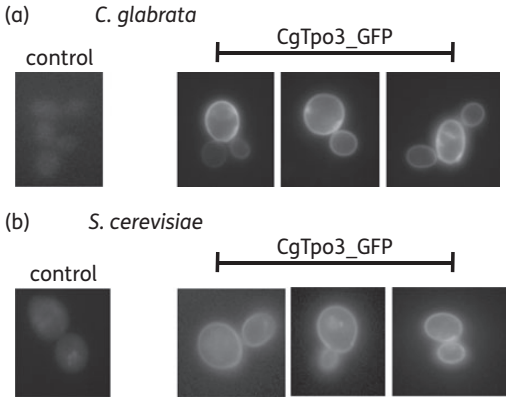

Figure 2. CgTpo3 localizes to the plasma membrane in *C. glabrata* and *S. cerevisiae* cells. Fluorescence of exponential phase L5U1 *C. glabrata* cells (a) or BY4741 *S. cerevisiae* cells (b), harbouring the cloning vector pGREG576 (control) or the pGREG576_*CgTPO3* or pGREG_MTI_*CgTPO3* plasmids (CgTpo3_GFP), after 5 h of galactose- or copper-induced recombinant protein production, respectively.

then transferred to the same medium containing 30 μM $CuSO_4$ to induce the expression of the fusion protein. At a standard OD_{600} 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 hydropathy 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 OD_{600} 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 [^{3}H]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 ^{3}H-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 [^{3}H]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 ^{3}H-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, [^{3}H]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,[13,14] 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

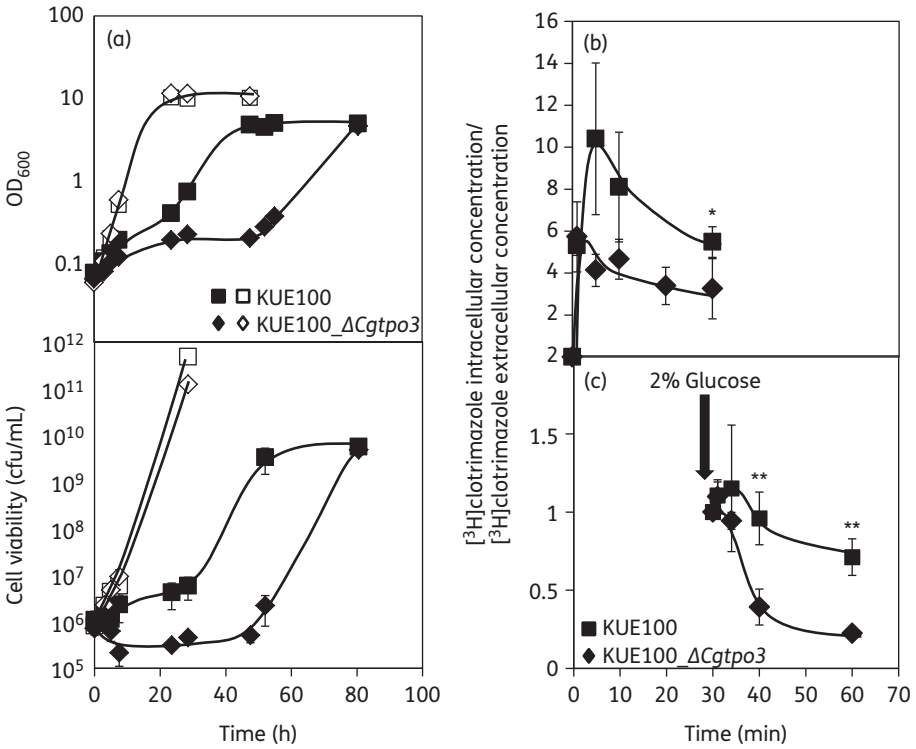

Figure 3. CgTpo3 expression increases *C. glabrata* resistance to clotrimazole by mediating the efflux of [^{3}H]clotrimazole in *C. glabrata*. (a) Comparison of the growth curves of *C. glabrata* KUE100 and KUE100_*ΔCgtpo3* strains, in liquid BM, in the absence (open symbols) or presence of 75 mg/L clotrimazole (filled symbols), measured in terms of variation in OD_{600} and in terms of variations in cell viability, determined as cfu/mL. The displayed growth curves are representative of at least three independent experiments. (b) Time-course accumulation ratio of [^{3}H]clotrimazole in non-adapted cells of KUE100 or KUE100_*Δcgtpo3* strains during cultivation in liquid BM in the presence of 30 mg/L unlabelled clotrimazole. (c) Time-course efflux ratio of [^{3}H]clotrimazole in pre-loaded cells of KUE100 or KUE100_*Δcgtpo3* upon a glucose pulse given after 30 min of passive accumulation of the radiolabelled drug. The accumulation ratio values are averages of at least three independent experiments. Error bars represent the corresponding standard deviations. $^*P<0.05$. $^{**}P<0.01$.

(Figure 5a). 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 *Δcgtpo3* 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 assessed for the KUE100 wild-type strain. Pre-exposure to 4 mM spermine for 1 h was indeed found to render *C. glabrata* cells more resistant to 25 mg/L clotrimazole in YPD medium when compared with non-exposed exponentially growing cells (Figure S3A, available as Supplementary data at *JAC* Online). By contrast, the simultaneous presence of 75 mg/L clotrimazole and a non-inhibitory concentration of spermine (2 mM) severely increased *C. glabrata* growth inhibition in BM when compared with exposure to clotrimazole alone (Figure S3B, available as Supplementary data at *JAC* Online). The different effects on growth inhibition of the concentrations of clotrimazole used in these assays result from the fact that they were carried out in 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), 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

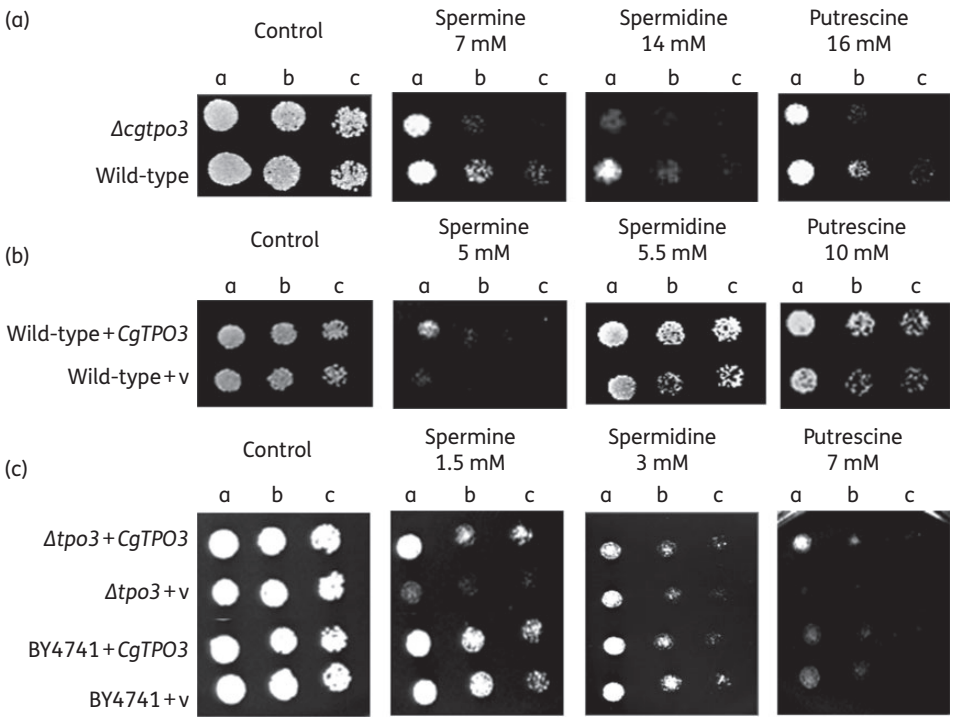

Figure 4. CgTpo3 confers resistance to azole antifungal drugs in *C. glabrata* and *S. cerevisiae* cells. (a) Comparison of the susceptibility to inhibitory concentrations of polyamines of the *C. glabrata* KUE100 and KUE100_*Δcgtpo3* strains on BM agar plates by spot assays. (b) Comparison of the susceptibility to inhibitory concentrations of polyamines of the *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 *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.

found to slightly increase *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 *C. glabrata* resistance towards this stress (Figure S4, available as Supplementary data at *JAC* Online).

Discussion

In this study, the functional characterization of the *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 *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, tioconazole, 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 *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 *CgCDR1* and *CgCDR2*.[25] Furthermore, the ABC drug efflux pump encoded by *CgAUS1* appears to function as a sterol transporter that may contribute to lower azole susceptibility in the presence of serum and to protect *C. glabrata* against azole toxicity *in vivo*.[26] The eventual participation of the DHA family members in imidazole resistance has been shown very recently to include in *C. glabrata* the action of the *CgQDR2* gene.[10] However, in this study, CgTpo3 was identified as the first of its family to confer fluconazole resistance on *C. glabrata*. Significantly, CgTpo3 has close homologues in other pathogenic *Candida* species, which may also play a role in azole drug resistance in these related pathogenic yeasts, including those encoded by *orf19.4737*, in *C. albicans*, *CPAR2_804310*, in *Candida parapsilosis*, and *Cd36_08290*, from *C. dubliniensis*.

Although many of the characterized drug efflux pumps have been shown to confer resistance to a wide variety of drugs and chemicals,[5,27,28] the molecular mechanisms behind their apparent promiscuity remains elusive.[4,5,28–31] Based on its high degree of homology and functional similarity to the *S. cerevisiae TPO3* gene, a possible physiological role linked to yeast survival in the presence of inhibitory polyamine concentrations[13] was investigated. Indeed, CgTpo3 expression did improve *C. glabrata* and *S. cerevisiae* fitness under inhibitory spermine and putrescine concentrations, and complemented the susceptibility phenotype exhibited by the *S. cerevisiae Δtpo3* deletion mutant. Furthermore, *C. glabrata* cells exposed to inhibitory concentrations of polyamines were found to accumulate this metabolite intracellularly. This accumulation was exacerbated in the *Δcgtpo3* cells,

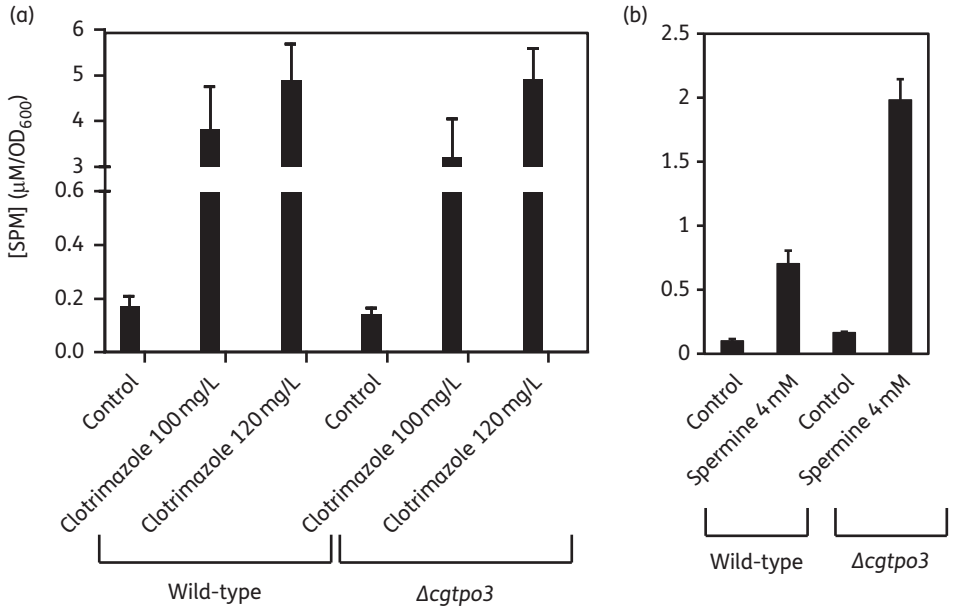

Figure 5. Intracellular accumulation of spermine in cells exposed to inhibitory concentrations of spermine, but not of clotrimazole, is dependent on CgTpo3. Comparison of the intracellular concentration of spermine in *C. glabrata* KUE100 and KUE100_Δ*cgtpo3* strains, cultivated in BM or YPD media, before (control) and upon exposure to inhibitory concentrations of clotrimazole (a) or spermine (b), respectively. The spermine concentration values, assessed as described in the Materials and methods section, are the averages of at least three independent experiments. Error bars represent the corresponding standard deviations. SPM, spermine.

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.[33] 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.[34] 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).[35] 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 pre-exposure 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 and azole 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 *CgTPO3* 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

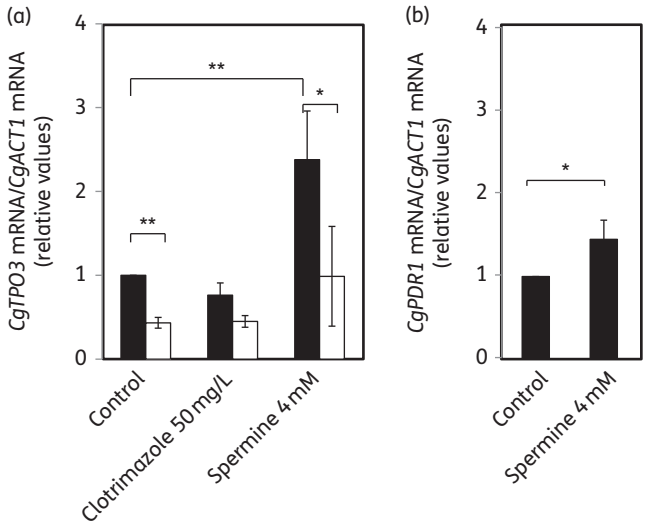

Figure 6. *CgTPO3* and *CgPDR1* transcript levels are up-regulated under spermine-induced stress. (a) Comparison of the variation of the *CgTPO3* transcript levels in wild-type (filled bars) or *Δcgpdr1* (open bars) *C. glabrata* cells before (control) and after 1 h of exposure to clotrimazole- or spermine-induced 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. *$P < 0.05$. **$P < 0.01$.

observation, based on microarray analysis, that in the absence of CgPdr1, the expression of *CgTPO3* is down-regulated.[38] An analysis of the *CgTPO3* promoter region, performed in the Regulatory Sequence Analysis Tools web site,[39] failed to identify any of the canonical CgPdr1 element (PDRE: BCCRYYRGD[6]). 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.

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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/).

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