New players controlling multidrug resistance and biofilm formation in *C. glabrata*: the important role of *Rpn4*

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Candida glabrata is the second most common pathogenic *Candida* species and has emerged as a leading cause of nosocomial fungal infections. Its reduced susceptibility to antifungal drugs and unusual ability to sustain proliferate within the human host make it an interesting research focus. However, little is known about the mechanisms underlying the transcriptional control of multidrug resistance and biofilm formation in this pathogen. Herein, a phenotypic study on seventeen uncharacterized *C. glabrata* ORFs, selected based on their amino acid similarity to *S. cerevisiae* or *C. albicans* known transcription factors was carried out in an attempt to identify new players in these processes. Antifungal susceptibility assays and biofilm formation assessment showed that *CgRPN4*, a predicted stimulator of proteasomal genes, is involved in both azole drug resistance and biofilm formation mechanisms. Its subcellular localization assessment through fluorescence microscopy demonstrated nuclear localization of the transcription factor in both cells under control conditions and cells undergoing fluconazole or ketoconazole exposure. The effect of the deletion of *CgRPN4* in *C. glabrata* transcriptome-wide response to fluconazole was also assessed using RNA-seq. CgRpn4 was found to activate the expression of genes involved, for instance, in amino acid metabolism, while repressing, probably indirectly, the expression of genes involved in ribosomal biogenesis and translation. These genes are promising candidates as new players in the context of azole resistance. The analysis of the promoter regions of the Rpn4 activated target genes enabled the identification of two possible parts of the binding site for this transcription factor: GAAGCA and AGTCTA. Altogether, this study highlights the complexity of the transcription regulatory networks that govern pathogenesis related phenotypes, reinforcing the need to obtain a complete picture of these processes to design more suitable tools to fight fungal infections.

Keywords: *Candida glabrata*, antifungal drug resistance, biofilm formation, *CgRPN4*, RNA-seq approach.

INTRODUCTION

Infections caused by fungal pathogens have become a relevant threat to human health as their prevalence has continuously increased over the past decades [1]. *Candida* species are the most common opportunistic fungal pathogens in humans, with *Candida albicans* remaining the predominant causative agent of all forms of candidiasis [2, 3]. Epidemiological data, however, indicate the growing role of non-albicans *Candida* (NAC) as causative agents of nosocomial invasive candidaemias, altogether surpassing *C. albicans* [4, 5]. Most infections attributed to NAC are caused by *Candida glabrata*, *Candida parapsilosis* and *Candida tropicalis*. This changing epidemiology and shift towards species characterized by elevated, as compared with *C. albicans*, MICs of azoles, reflects their widespread use and prolonged prophylaxis in the growing population of high-risk patients [6].

Until recently, *Candida glabrata* was considered a relatively nonpathogenic commensal fungal organism of human mucosal tissues. In fact, although belonging to *Candida* genus, it is phylogenetically closer to *S. cerevisiae*. Nonetheless, with the increased use of immunosuppressive agents, mucosal and systemic infections caused by *C. glabrata* have increased significantly, especially in the human immunodeficiency virus-infected population [7]. A major obstacle in *C. glabrata* infections treatment is their innate resistance to azole antimycotic therapy, which is very effective in treating infections caused by other *Candida* species. Mortality rates are higher in *C. glabrata* than in *C. albicans* infections, with an average mortality rate of 50% (30%-80%) [8]. The occurrence of *C. glabrata* in such diverse habitats such as gastrointestinal tract, vaginal mucosa, skin and blood, suggests that it has the ability to adapt efficiently to changes in the environment. Additionally, the importance of both species has increased dramatically because of their ability to form biofilms on different types of implant [9]. Despite their structural diversity, biofilm, formation by both *Candida* species is influenced by the choice of biomaterial, medium and carbohydrate source and concentration. Additionally, pH plays an important role, as *Candida spp*. may colonize different niches with respect to the environmental pH [10, 11]. The major problem in treating biofilm-associated infections is the resistance of the fungal population to most classes of antifungals. Therefore, it is crucial to understand the mechanisms underlying the multidrug resistance phenomenon on *candida* species, specially *C. glabrata* due to its innate resistance to azole antimycotic therapy, as well as the mechanisms that lead to biofilm formation, which increases this human pathogen resistance to administrated antifungal drugs, and the mechanisms of oxidative stress response, which are required to tolerate high concentrations of reactive oxygen species (ROS) produced by the host as a defense mechanism.

Herein, a phenotypic screening on seventeen uncharacterized *C. glabrata* predicted transcription factors (TF) was undertaken in an attempt to identify new players in the transcriptional control of multidrug resistance and biofilm formation in this human pathogen. This analysis led to functional characterization of *CgRPN4*, with emphasis on its role in antifungal drug resistance. This gene encodes a putative TF for proteasomal genes, sharing a high degree of homology with the *S. cerevisiae RPN4* gene. The last is a TF that stimulates expression of proteasomal genes and is transcriptionally regulated by the pleiotropic drug resistance regulator *PDR1*. In this study, CgRpn4 was found confer resistance to azole drugs. Additionally, the effect of *CgRPN4* deletion in the transcriptome-wide response of *C. glabrata* cells toward mild fluconazole-induced stress conditions was assessed through RNA-sequencing.

MATERIALS AND METHODS

Strains and plasmids

Saccharomyces cerevisiae parental strain BY4741 (*MATa, ura3Δ0, leu2Δ0, his3Δ1, met15Δ0*) and derived single deletion mutant BY4741_ *Δrpn4* were obtained from Euroscarf collection. *C. glabrata* parental strain KUE100 [12] and derived single deletion mutants *Δcgrpn4, Δcgstb5, Δcgpdr1, Δcgyrm1_1, Δcgyrm1_2, Δcghap1, Δcgmrr1, Δcghal9, Δcgtac1, Δcgskn7, Δcgyap1, Δcgcad1, Δcgtog1, Δcgtec1_1, Δcgtec1_2, Δcgbcr1* and *Δcgndt80* were kindly provided by Hiroji Chibana, from the Medical Research Center (MMRC), Chiba University, Chiba, Japan. *C. glabrata* strain L5U1 (*cgura3Δ0, cgleu2Δ0*) was kindly provided by John Bennett from the National Institute of Allergy and Infectious Diseases, NH, Bethesda, USA. Also, CBS138 C. glabrata strain was used in this study for gene amplification purposes. The plasmids pGREG576 and 515 were obtained from Drag & Drop collection [13].

Antifungal susceptibility assays

C. glabrata cells were batch-cultured at 30°C, with orbital agitation (250 rpm), in minimal medium without supplementation (MMB), containing (per liter): 1.7 g of yeast nitrogen base without amino acids or NH_4^+ (Difco), 20 g of glucose (Merck) and 2.7 g of $(NH_4)_2SO_4$ (Merck). The cell suspensions used to inoculate the agar plates were mid-exponential cells grown until a standard culture final $OD_{600\ nm}$ = 0.4 ± 0.02 was reached and then diluted in sterile water to obtain suspensions with final $OD_{600\ nm}$ = 0.05 ± 0.005. These cell suspensions and subsequent dilutions (1:5; 1:25) were applied as 4 µL spots onto the surface of solid MMB medium, supplemented with adequate chemical stress concentrations. Agarized solid media contained, besides the previously indicated ingredients, 20 g/L agar (Iberagar).

Additionally, minimal inhibitory concentration (MIC) assays were performed using standardized concentrations of ketoconazole, fluconazole (azoles), amphotericinB (polyene), flucytosine (pyrimidine) and caspofungin (echinocandin). The MIC (minimum inhibitory concentration) was defined as the lowest drug concentration inhibiting growth at least 50% relative to the drug-free control, called MIC 50% (MIC_{50}). Cells were grown at 30°C, with orbital agitation (250 rpm), in liquid rich medium YPD and the assays were performed as described by Rodríguez-Tudela *et al.* (2003) [14], using a standard cellular suspension with final $OD_{600\ nm}$ = 0.05 ± 0.005. The microtiter plates were incubated without agitation at 37°C for 24 hours and then were read in a microplate reader (SPECTROstar Nano from BMG LabTech) using a wavelength of 530 nm.

Biofilm quantification assays

Cells were batch-cultured at 30ºC, with orbital agitation (250 rpm) in double-strength Sabouraud Dextrose Broth (SDB) medium, at pH 5.6, containing (per liter): 80 g glucose (Merck) and 20 g meat peptone (Merck). The double-strength RPMI 1640 media used to grow the cells in the microtiter plates is composed (per 300 mL) by: 6.24 g RPMI 1640 (Sigma), 20.72 g MOPs (Sigma) and 10.8 g glucose (Merck). The pH was adjusted using a HCl 1M solution. Cells were collected at mid-exponential phase and a cellular suspension with final $OD_{600\ nm}$ = 0.1 was prepared to inoculate a 96-well polystyrene titter plates (Greiner), which were previously filled with the appropriated growth medium in order to have an initial OD_{600nm} = 0.05 ± 0.005. The microtiter plates were incubated at 30°C, with mild orbital agitation (70 rpm) for 16h. Biofilm quantification was assessed using crystal-violet method as described by Pathak and colleagues (2012) [15] and the results were obtained using a microplate reader (SPECTROstar Nano, BMG LabTech) at 590 nm.

S. cerevisiae and C. glabrata transformation

Cells were batch-cultured at 30°C, with orbital agitation (250 rpm) in liquid rich medium Yeast extract–Peptone-Dextrose (YPD), with the following composition (per liter): 20 g of glucose (Merck), 20 g of bacterial peptone (Dickson) and 10 g of yeast extract (HIMEDIA). Mid-exponential *S. cerevisiae* BY4741 and *C. glabrata* L5U1 cells were once again batch-cultured at 30ºC with orbital shaking (250 rpm) in YPD liquid medium until a standard OD_{600nm} 0.4 ± 0.04 was reached. Cells were harvested and the transformation reactions were performed using the Alkali-Cation Yeast Transformation Kit (MP Biomedicals), according to the manufacturer's instructions. Transformed cells were plated in appropriate medium agarized plates.

*Cloning of the C. glabrata CgRPN4 (*ORF *CAGL0K01727g) gene under the control of the MTI promoter*

The pGREG576 plasmid from the Drag & Drop collection [13] was used to clone and express the *C. glabrata* ORF *CAGL0K01727g* in *S. cerevisiae*, as described before for other heterologous genes [16]. It contains a galactose inducible promoter (*GAL1*), the yeast selectable marker *URA3* and the *GFP* gene, encoding a Green Fluorescent Protein (GFP^{S65T}), which allows monitoring of the expression and subcellular localization of the cloned fusion protein. The plasmid was restricted with the restriction enzyme *SalI* (Takara) in the cloning site harboring the *HIS3* gene. Additionally, it was treated with *CiAP* (Invitrogen) during 45 minutes at 37ºC to prevent recircularization. The *CAGL0K01727g* DNA was generated by PCR, using genomic DNA extracted from the sequenced CBS138 *C. glabrata* strain using the specific primers: 5'-GAATTCGATATCAAGCTTATCG ATACCGTCGACA*ATGACGTCTATAGATTTGGGAC*-3' and 5'-GCGTGACATAACTAATTACATGACTCGAGGTCGAC*TTAT GCAGTGACAAATCCGATG*-3'. The designed primers contain, besides a region with homology to the first and the last 22 nucleotides of the *CAGL0K01727g* coding region (italic), nucleotide sequences with homology to the cloning site flanking regions of the pGREG576 vector (underlined). The amplified DNA fragment was co-transformed into the parental *S. cerevisiae* BY4741 with the pGREG576 vector, previously cut with the restriction enzyme *SalI* (Takara), generating pGREG576_*CgRPN4* plasmid. Co-transformed *S. cerevisiae* cells were plated in minimal medium supplemented with 20 mg/L histidine, 20 mg/L methionine and 60 mg/L leucine (MMB-U), containing (per liter): 1.7 g of yeast nitrogen base without amino acids or NH4+ (Difco), 5 g of glucose (Merck), 1 g of galactose (Sigma), 2.7 g of $(NH_4)_2SO_4$ (Merck) and 20 g/L agar (Iberagar). Since the *GAL1* promoter only allows a slight expression of downstream genes in *C. glabrata*, it was replaced by the copper-induced *MTI C. glabrata* promoter, originating the pGREG576_*MTI_CgRPN4* plasmid. *MTI* promoter DNA was generated by PCR, using genomic DNA extracted from CBS138 *C. glabrata* strain, and the specific primers: 5'-TTAACCCTCACTAAAGGGAACAAAAGCTGGAGCTC*TGTA CGACACGCATCATGTGGCAATC* – 3'and 5' – GAAAAGTTCT TCTCCTTTACTCATACTAGTGCGGC*TGTGTTTGTTTTTGTA TGTGTTTGTTG* - 3'. The designed primers contain, besides a region with homology to the first 26 and the last 27 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_*CgRPN4* plasmid, previously cut with *SacI* and *NotI* (Takara) restriction enzymes to remove the *GAL1* promoter, generating the pGREG576_*MTI_CgRPN4* plasmid.

CgRpn4 subcellular localization assessment

The subcellular localization of the CgRpn4 protein was determined based on the observation of L5U1 *C. glabrata* cells transformed with the pGREG576_*MTI_CgRPN4* plasmid,

expressing the CgRpn4_GFP fusion protein. Cell suspensions were prepared in minimal medium supplemented with 60 mg/L leucine (MMG-U), until a standard culture OD_{600nm} = 0.4 ± 0.04 was reached, and transferred to the same medium MMG-U supplemented with 50 µM $CuSO_4$ (Sigma), to induce protein overexpression. After 6h of incubation, 150 mg/L fluconazole or 40 mg/L ketoconazole were added to the culture and cells were harvested after 1h of exposure. As control samples, culture grown in the absence of antifungal drugs were also inspected. 2 mL of cell suspension were centrifuged at 13 500 rpm for 2 minutes, and the pelleted cells were resuspended in 5 µL distilled water. The distribution of CgRpn4_GFP fusion protein in *C. glabrata* living cells was detected by fluorescence microscopy in a Zeiss Axioplan microscope (Carl Zeiss MicroImaging), using excitation and emission wavelength of 395 and 509 nm, respectively. Fluorescence images were captured using a cooled CCD camera (Cool SNAPFX, Roper Scientific Photometrics).

Antifungal susceptibility assays in S. cerevisiae and C. glabrata cells overexpressing CgRPN4

The ability of *CgRPN4* gene expression to increase *S. cerevisiae* and *C. glabrata* cells resistance toward azole drugs was assessed, through spot assays, in the *URA3*$^-$ strains BY4741 *S. cerevisiae* and L5U1 *C. glabrata*, using pGREG576_*CgRPN4* and pGREG576_*MTI_CgRPN4* centromeric plasmids, respectively. Additionally, the capability of *CgRPN4* gene expression to complement the absence of its *S. cerevisiae* homolog (*ScRPN4*) in BY4741_*Δrpn4* was also assessed through spot assays, using pGREG576_*CgRPN4*. *S. cerevisiae* cells were first batch-cultured in MMB-U 0.5% glucose and 0.1% galactose medium, at 30°C, with orbital agitation (250 rpm) until a standard culture OD_{600nm} = 0.4 ± 0.04 was reached. Then, the cells from this first culture were used to initiate a new batch-culture, with an initial OD_{600nm} = 0.1 ± 0.01, in MMB-U 0.1% glucose and 1% galactose medium at 30°C, with orbital agitation (250 rpm). *C. glabrata* cell suspensions were prepared in MMG-U, at 30°C, with orbital agitation (250 rpm), until a standard culture OD_{600nm} = 0.4 ± 0.04 was reached. Then, the cells were transferred, with an initial OD600nm = 0.1 ± 0.02, to the same medium MMG-U supplemented with 50 µM $CuSO_4$ (Sigma), to induce protein overexpression. Cell suspensions used to inoculate the agar plates were mid-exponential cells grown until culture OD_{600nm} = 0.4 ± 0.02 was reached and then diluted in sterile water to obtain suspensions with OD^{600nm} = 0.05 ± 0.005. These *S. cerevisiae* and *C. glabrata* cell suspensions and subsequent dilutions (1:5; 1:25) were applied as 4 µL spots onto the surface of solid MMB-U 0.1% glucose and 1% galactose or MMG-U with 50 µM CuSO4, respectively. The plates were supplemented with adequate stress concentrations of the azole antifungal drugs ketoconazole (30 to 40 mg/L for *C. glabrata*, and 15 to 20 mg/L for *S. cerevisiae*) and fluconazole (100 to 150 mg/L *C. glabrata*, and 60 to 80 mg/L for *S. cerevisiae*).

RNA-sequencing analysis

C. glabrata cells for RNA-seq analysis were grown in MMB, at 30°C, with orbital agitation (250 rpm), until mid-log phase. Subsequently, 250 mg/L fluconazole were added to the culture and cells were harvested after 1h. For control samples, no fluconazole was added to the culture. Total RNA was isolated using an Ambion Ribopure-Yeast RNA kit, according to manufacturer's instructions. Strand specific RNA-seq library preparation and sequencing was carried out as a paid service by the NGS core from Oklahoma Medical Research Foundation, Oklahoma City, Oklahoma, USA. Paired-end reads (Illumina HiSeq 3000 PE150, 2x150 bp, 2 GB clean data) were obtained from wild type *C. glabrata* KUE100 and correspondent deletion mutant strain KUE100_*Δcgrpn4* (ORF *CAGL0K01727g*). Two replicates of each sample were obtained from three independent RNA isolations, subsequently pooled together. Sample reads were trimmed using Skewer [17] and aligned to the *C. glabrata* CBS138 reference genome, obtained from the *Candida* Genome Database (CGD) (http://www.candidagenome.org/), using TopHat [18]. HTSeq [19] was used to count mapped reads per gene. Differentially expressed genes were identified using DESeq2 [20] with an adjusted p-value threshold of 0.01 and a $\log_2$ fold change threshold of -1.0 and 1.0. Default parameters in DESeq2 were used. *C. albicans* and *S. cerevisiae* homologs were obtained from the CGD and *Saccharomyces* Genome Database (SGD) (https://www.yeastgenome.org/), respectively. The GO term finder from CGD [21] was used to carry out Gene Ontology (GO) analyses.

RESULTS

Antifungal susceptibility assays

The difference in the susceptibility of *C. glabrata* KUE100 wild-type strain and seventeen derived single deletion mutants toward inhibitory concentrations of several chemical stress inducers was accessed through spot assays (Fig. 1) and, in the case of selected antifungal drugs, confirmed by MIC assays.

Concerning the *C. glabrata* ORFs predicted to play a role in multidrug resistance, some were found to, indeed, confer antifungal drug resistance. For instance, the deletion of *CgPDR1*, *CgRPN4* or *CAGL0L04576g* (*CgYRM1_1*) was found to increase the susceptibility of *C. glabrata* cells toward all azole antifungal drugs tested, whereas the deletion of *CgSTB5* or *CAGL0L04400g* (*CgYRM1_2*) was found not to affect *C. glabrata* susceptibility to any of the tested antifungal drugs. In fact, *CgPDR1* demonstrated to be crucial for *C. glabrata* resistance to several azole antifungal drugs, since the corresponding deletion mutant *Δcgpdr1* was unable to growth in the presence of several azoles used. The key role of *CgPDR1* in *C. glabrata* azole resistance was further confirmed by MIC determination, through which the KUE100_ *Δcgpdr1* mutant was found to exhibit an 8-fold difference in terms of MIC levels for fluconazole and ketoconazole, relatively to the wild-type strain. Studies in the related yeast *S. cerevisiae* have shown that Pdr1p forms a heterodimer with the transcription factor, Stb5p [22]. In *C. glabrata*, it was demonstrated that many of the genes upregulated by overexpression of *CgPDR1* were upregulated by deletion of *CgSTB5* [23]. Additionally, the overexpression of *CgSTB5* was shown to repress azole resistance, whereas it's deletion caused a modest increase in resistance [22]. As displayed in Figure 1, although the *Δcgstb5* mutant presented reduced growth in the control conditions when compared with the wild-type strain, there was no increase in susceptibly of this mutant in the presence of several azole antifungal drugs or even toward amphotericin B or flucytosine. However, there seems to be a slight increase of this mutant resistance toward some of the azoles tested, such as tioconazole and miconazole, but these differences were not further analyzed here. MIC assays showed no differences in terms of MIC levels for fluconazole, ketoconazole, amphotericin B or flucytosine between *Δcgstb5* mutant and the wild-type strain. The deletion of *CgRPN4* was found to increase the susceptibility of *C. glabrata* toward all azole drugs tested. This was confirmed by MIC assays, for fluconazole and ketoconazole, with a 4-fold difference in terms of MIC levels between the wild-type and the *Δcgrpn4* mutant strain. These results suggest a clear role of *CgRPN4* encoded protein as multidrug resistance determinant protein in *C. glabrata*. Also, the deletion of *CAGL0L04576g* (*CgYRM1_1*) seemed to increase the mutant susceptibility toward some azoles, mainly to ketoconazole. However, the fluconazole and ketoconazole MIC levels exhibited by the *Δcgyrm1_1* deletion mutant was found to be similar to those displayed by the wild-type strain.

Within the *C. glabrata* ORFs whose closest *S. cerevisiae* homologs play a role in oxidative stress response, none was found to play a role in *C. glabrata* resistance toward azole drugs, since all the mutants grew as the wild-type strain in the presence of several azole inhibitory concentrations. Yet, the deletion of *CgYAP1* was

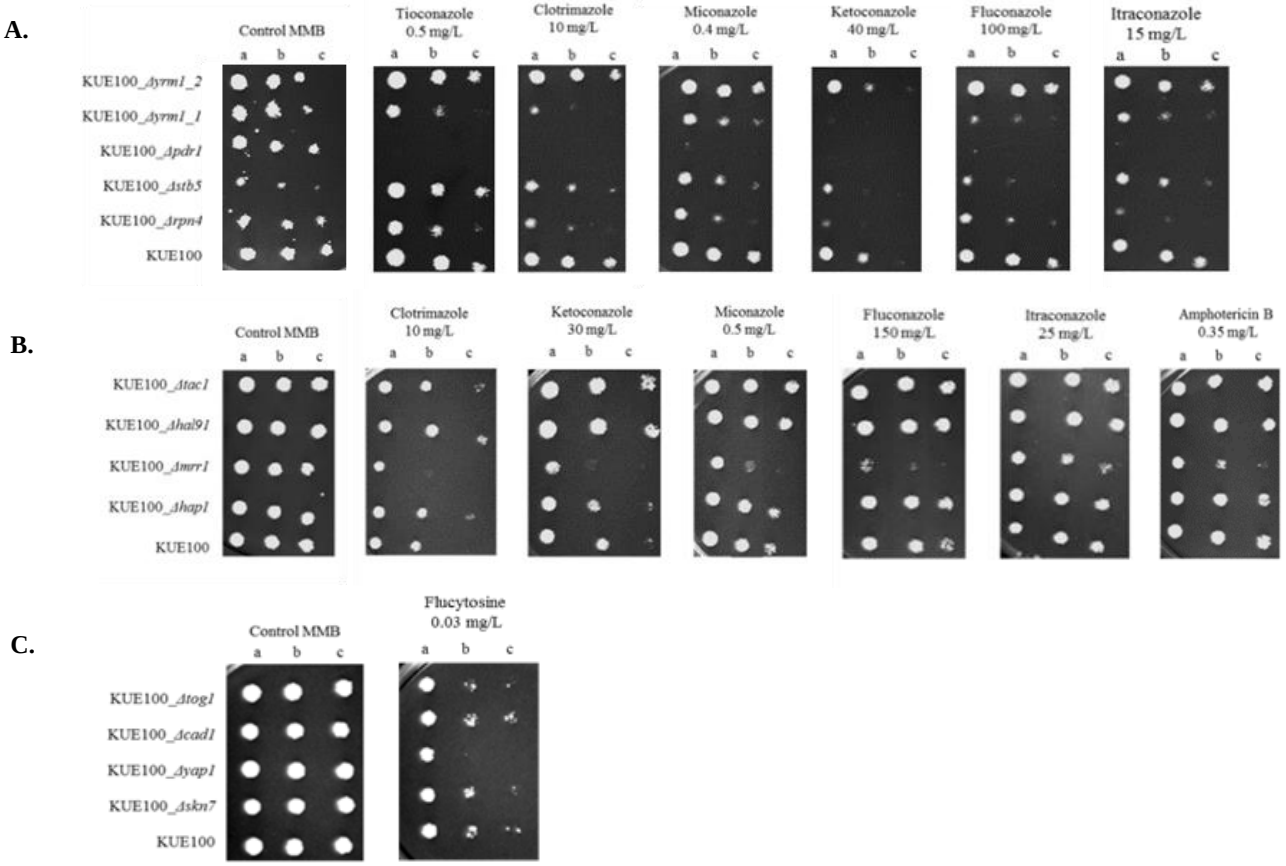

Figure 1 | Susceptibility assays. Comparison of the susceptibility to inhibitory concentrations of several chemical stress inducers, at the indicated concentrations, of the *C. glabrata* KUE100, (A) KUE100_*Δrpn4*, KUE100_*Δstb5*, KUE100_*Δpdr1*, KUE100_*Δyrm1_1* and KUE100_*Δyrm1_2*; (B) KUE100_*Δhap1,* KUE100_*Δmrr1,* KUE100_*Δhal9,* and KUE100_*Δtac1*; (C) KUE100_*Δskn7,* KUE100_*Δyap1,* KUE100_*Δcad1* and KUE100_*Δtog1* strains in MMB agar plates by spot assays. The inocula were prepared as described in "Material and methods" for spot assays. Cell suspensions used to prepare the spots were 1:5 (b) and 1:25 (c) dilutions of the cell suspension used in (a). The displayed images are representative of three independent experiments.

found to slightly increase the susceptibility of *C. glabrata* to flucytosine, although no difference between fluconazole and ketoconazole MIC levels was found between the *Δcgyap1* deletion mutant and its parental strain.

The deletion of *CAGL0B03421g* (*CgMRR1*), the closest homolog to *C. albicans MRR1*, encoding an azole resistant determinant in this species, was found to increase the susceptibility of *C. glabrata* to all azole antifungal drugs tested. Additionally, *Δcgmrr1* mutant presented slightly increased susceptibility toward other antifungal drug families, namely to the polyene amphotericin B. As it is clear in Figure 1, the wild-type strain grew in the tested drug concentrations, while the *Δcgmrr1* mutant displayed growth limitation when exposed to all azoles tested and also to amphotericin B. On one hand, MIC assays confirmed the increased susceptibility of *Δcgmrr1* mutant toward fluconazole and ketoconazole, with a 2-fold difference in terms of MIC levels, when compared to the wild-type. On the other hand, no differences in amphotericin B susceptibility were found between the mutant and the wild-type strains.

Within the *C. glabrata* ORFs whose closest *C. albicans* homologs play a role in biofilm formation, none was found to confer resistance to the tested antifungal drugs.

In order to determine if any of the *C. glabrata* ORFs under analysis are involved in this pathogen resistance toward echinocandins, MIC assays were performed to compare the single deletion mutant's susceptibility toward caspofungin relatively to the wild-type strain. Only *Δcgrpn4* mutant displayed increased susceptibility when exposed to inhibitory concentrations of caspofungin, presenting a 2-fold difference in terms of MIC levels comparing to the wild-type.

Biofilm quantification assays

In order to screen for novel regulators of biofilm development, biofilm formation was assessed in the *C. glabrata* parental strain KUE100 and in *Δrpn4*, *Δstb5*, *Δyrm1_1*, *Δyrm1_2, Δtog1*, *Δhap1*, *Δmrr1*, *Δtac1*, *Δyap1*, *Δskn7* and *Δcad1* derived single deletion mutants as described before in Section 2.2.2. Most of the strains tested showed greater biofilm production when the cells were grown in RPMI 1640 pH 4 rather than in SDB pH 5.6. In fact, previous studies reported that *C. glabrata* cells formed significantly thicker biofilms in RPMI 1640 medium compared with others, and perceives low pH as less stressful than higher pH, contrasting with *C. albicans*, which reinforce the greater biofilm production in RPMI 1640 pH 4 rather than in SDB pH 5.6 [24]. Additionally, SDB medium is less nutrient-rich than RPMI 1640 medium which mimics the composition of human fluids [25]. According to the results obtained, *Δcghap1, Δcgmrr1, Δcgyap1, Δcgcad1* and *Δcgskn7* mutants presented significant ($p<0.05$) decreased biofilm formation in both RPMI and SDB medium. *Δcgtog1*, *Δcgyrm1_2,* and *Δcgstb5* mutant strains presented significantly increased biofilm formation when cells were cultured in SBD comparing to the wild-type, but not in RPMI. In contrast, *Δcgrpn4* and *Δcgtac1* mutants displayed greatly decreased biofilm production in SDB whereas, in RPMI, the values equal or even exceeds those for wild-type.

CgRpn4 expression and subcellular localization in C. glabrata

To address the issue of the CgRpn4 activation mechanisms, subcellular localization of CgRpn4p was assessed, using a GFP fusion, in *C. glabrata* cells. The localization of this transcription factor, in cells under control conditions, was compared to that in

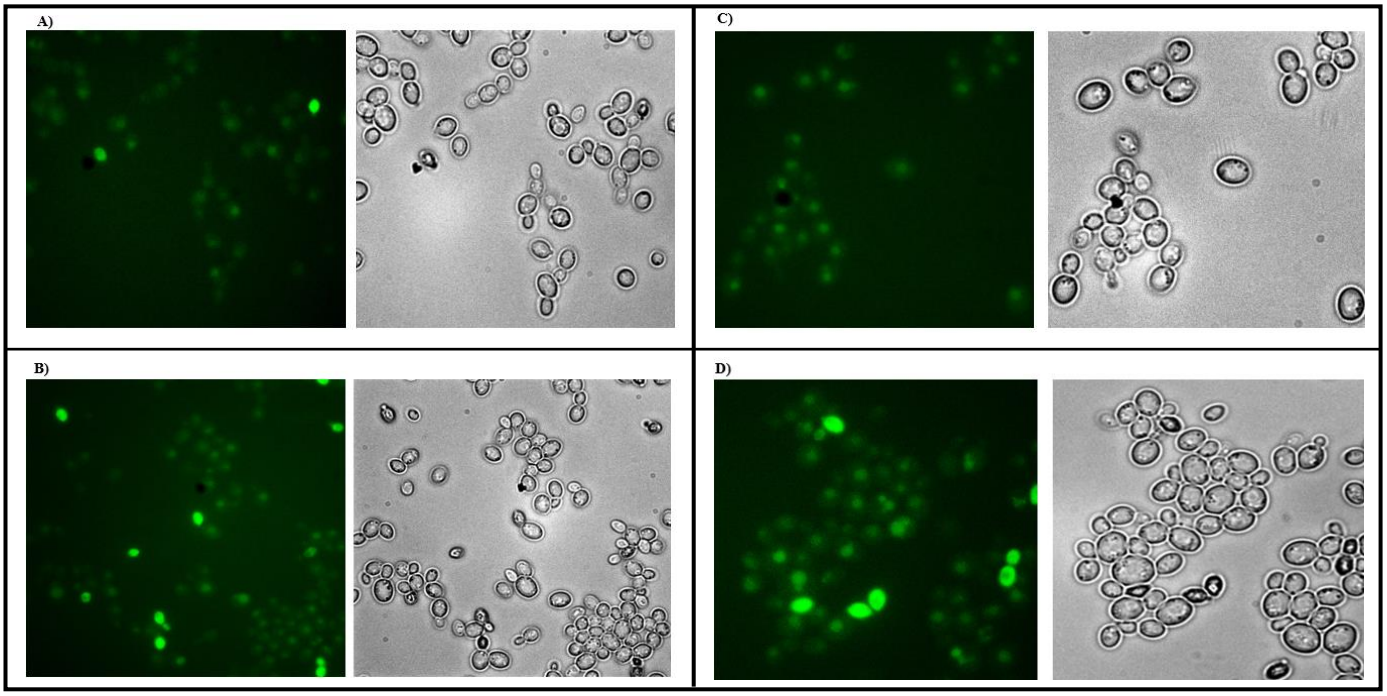

Figure 2 | Fluorescence of exponential phase L5U1 *C. glabrata* cells harboring the pGREG_*MTI_CgRPN4* plasmid, after copper-induced recombinant protein production under control conditions, in exponential **(A)** and stationary **(B)** phase, and under fluconazole-induced **(C)** or ketoconazole-induced stress conditions **(B)**.

cells undergoing antifungal drug exposure. *C. glabrata* cells expressing CgRpn4_GFP fusion protein were analyzed by fluorescence microscopy and nuclear localization of the transcription factor was verified in both cells under control conditions and cells undergoing fluconazole or ketoconazole exposure. To confirm that CgRpn4p nuclear localization verified in cells under control conditions was not a consequence of those cells being early stationary phase cells, its localization in exponential *C. glabrata* cells also was assessed. As displayed in Figure 2, CgRpn4p presents nuclear localization in all the tested conditions. In order to sustain these observations, a quantitative analysis was undertaken to determine the ratio of cells with transcription factor nuclear localization relatively to the total cells presenting fluorescence in different preparations of each condition tested. A total of ± 400 *C. glabrata* fluorescent cells was analyzed per condition. Concerning control conditions, as displayed in Figure 3, only 57.5% of exponential phase cells analyzed presented CgRpn4 nuclear localization, in contrast with stationary phase cells in which more than 85% of the cells showed pronounced transcription factor nuclear localization. Under azole-induced stress conditions, the transcription factor remained in the cell's nucleus, with 90% of both fluconazole and ketoconazole-stressed cells presenting nuclear CgRpn4 accumulation. These results suggest that, although

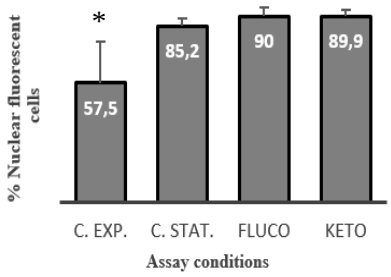

Figure 3 | Fraction of *C. glabrata* cells presenting nuclear localized fluorescence. L5U1 *C. glabrata* cells harboring the pGREG_*MTI_CgRPN4* plasmid, after copper-induced recombinant protein production, were analyzed by fluorescence microscopy under control conditions and under fluconazole-induced or ketoconazole-induced stress conditions. In the bar chart, each bar corresponds to the percentage of cells presenting nuclear localized fluorescence from a total of ± 400 control stationary phase, control exponential phase, fluconazole-stressed and ketoconazole-stressed cells. *p<0.05.

a large proportion of the population displays CgRpn4 accumulation in the nucleus, there is a certain degree of increased nuclear accumulation of CgRpn4 under stress, both that induced by antifungal drugs or by reaching stationary phase of growth. These findings represent a first step into the functional characterization of the *C. glabrata* putative transcription factor for proteasome genes CgRpn4, its nuclear localization, especially in cells underwent azole drug exposure, enhances its predicted role as stimulator of proteasomal gene expression transcriptionally regulated in response to environmental stresses.

CgRpn4 expression confers resistance to azole antifungal drugs

It was shown that the deletion of *CgRPN4* dramatically increases the susceptibility toward the azole antifungal drugs. Therefore, the effect of *CgRPN4* overexpression in *C. glabrata* cells azole resistance was assessed using inhibitory concentrations of fluconazole or ketoconazole. As it is clear in Figure 4, both *C. glabrata* cells harboring the cloning or the recombinant plasmid grew similarly in control conditions, with the L5U1 cells carrying the recombinant plasmid exhibiting a slightly slower growth when compared to those harboring the cloning vector. This might be due to the metabolic weight caused by the overexpression of the *CgRPN4*. However, when the cells were grown in the presence of inhibitory concentrations of the azole antifungals, only L5U1 cells overexpressing *CgRPN4* were able to grow. In fact, *C. glabrata* cells harboring the cloning vector were completely unable to growth in any of the antifungal-induced stress conditions tested. These results reinforce the key role of the transcription factor CgRpn4 as a key regulator of azole drug resistance in *C. glabrata*. Additionally, the capability of *CgRPN4* gene expression to complement the absence of its *S. cerevisiae* homolog (*ScRPN4*) in BY4741_*Δrpn4* mutant cells was assessed with lower drug concentrations, since *C. glabrata* displays intrinsically higher resistance to azoles than *S. cerevisiae*. As depicted in Figure 4, both wild-type and mutant strains harboring the cloning or recombinant plasmid grew similarly in control conditions, without antifungal drug exposure. On one hand, as expected, the growth of BY4741_*Δrpn4* cells carrying the cloning vector was strongly or even fully affected when the cells were grown in the presence of inhibitory concentrations of both fluconazole and ketoconazole, whereas the cells from the same strain harboring the recombinant

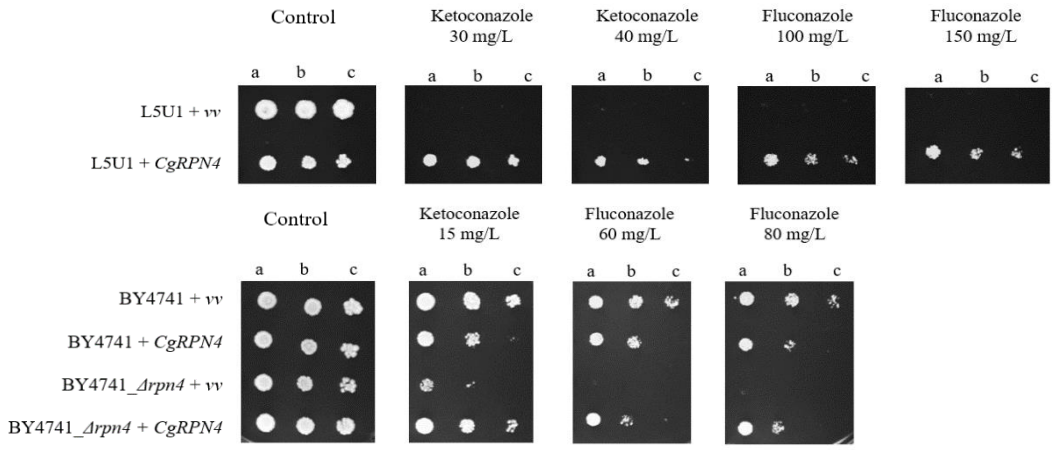

Figure 4 | *CgRPN4* overexpression influence in susceptibility. Comparison of the susceptibility to inhibitory concentrations of fluconazole or ketoconazole, at the indicated concentrations, of the *C. glabrata* L5U1 strain, harboring the pGREG576 cloning vector (*vv*) or the pGREG576_*MTI_CgRPN4 (CgRPN4*) plasmid; of the *S. cerevisiae* BY4741strain, harboring the pGREG576 cloning vector (*vv*) or the pGREG576_*CgRPN4* (*CgRPN4*) plasmid; and of the *S. cerevisiae* BY4741_*Δrpn4*, harboring the pGREG576 cloning vector (*vv*) or the pGREG576_*CgRPN4* (*CgRPN4*) plasmid through spot assays. The inocula were prepared as described in "Materials and Methods". Cell suspensions used to prepare the spots were 1:5 (b) and 1:25 (c) dilutions of the cell suspension used in (a). The displayed images are representative of at least three independent experiments.

plasmid were able to grow in those conditions. These results demonstrate that *CgRPN4* gene expression is able to complement the absence of its *S. cerevisiae* homolog (*ScRPN4*) in *S. cerevisiae* cells, suggesting that *ScRPN4* and *CgRPN4* are orthologous genes. On the other hand, surprisingly, the overexpression of *CgRPN4* in BY4741 *S. cerevisiae* wild-type cells does not seem to bring any advantage concerning this organism drug resistance. BY4741 cells harboring the cloning vector demonstrated a slightly lower susceptibility toward inhibitory concentrations of both fluconazole and ketoconazole when compared to those overexpressing *CgRPN4*, through the recombinant plasmid.

RNA-sequencing analysis

The effect of *CgRPN4* deletion in the transcriptome-wide response of *C. glabrata* cells to mild fluconazole-induced stress conditions was assessed to compare the genes up or downregulated in the *Δcgrpn4* deletion mutant relatively to the wild-type strain, in the same conditions (control or mild fluconazole-induced inhibition). However, the drug concentration was not sufficiently inhibitory to induce a strong defense response in wild-type cells. Nonetheless, the results obtained concerning the genes that are up or downregulated in the *Δcgrpn4* single deletion mutant compared to the wild-type strain, in both control and fluconazole-induced stress conditions, enabled the identification of a short list of genes whose expression was seen to depend on the CgRpn4 transcription factor. Altogether, CgRpn4 was found to activate the expression of 9 genes while repressing, possibly indirectly, 32 genes. The ORFs found to be regulated by CgRpn4, either in control conditions or mild fluconazole-induced stress conditions, were grouped into predicted functional clusters based on the function of their *S. cerevisiae* closest homologs, or in some cases based on the description of the *C. glabrata* ORFs (Table 1.). Among the CgRpn4 upregulated genes, three *C. glabrata* ORFs (*CAGL0M08552g*, *CAGL0D05214g* and *CAGL0D04840g*) have a close *S. cerevisiae* homolog known to be involved in azole resistance.

Table 1 | Major functional groups found to have significant expression changes in *C. glabrata CgRPN4* deletion mutant relatively to wild-type under control or mild fluconazole-induced stress conditions.

Control	Functional clusters	Fluconazole-induced stress
37%	RNA metabolism	29%
26%	Unknown function	24%
5%	Protein trafficking	11%
5%	Mitochondrial regulation	10%
16%	Plasma membrane organization	8%
11%	Metal homeostasis	5%
-	Amino acid metabolism	3%
-	Ribosomal biogenesis and translation	10%

To further analyze if the influence of CgRpn4 on the ORFs found to be downregulated in the *Δcgrpn4* mutant is through direct binding of the transcription factor to the ORFs promoter regions, those regions were analyzed using RSAT: oligo-analysis to search for binding motifs. After analyzing the promoter regions of the 6 CgRpn4 upregulated ORFs, the software found a total 17 different enriched motifs with lengths from 6 to 8 nucleotides. The same motif can be found several times in the same promoter region, meaning that the number of occurrences does not necessarily demonstrate that a specific binding site is present in more sequences of the input than another. Once having the transcription factor binding motifs predicted to exist in the upstream region of each CgRpn4 upregulated ORFs, a search for conserved consensus sequences of *S. cerevisiae* Rpn4 known binding motifs was performed based on the predicted oligomers. The longest consensus sequence found in these predicted motifs that might correspond to a conserved part of known ScRpn4 binding motifs was CCAC. This 4-nucleotide long sequence was found in 4 differently predicted binding motifs, although with different associated-significance. However, 4 nucleotide length consensus sequence has a high random nucleotide distribution associated. Within the 17 different binding motifs found to be enriched in CgRpn4 upregulated ORFs promoter regions, GAAGCA and AGTCTA were found in 5 of the 6 inputted upstream regions, suggesting that these consensus sequences may be part of CgRpn4 binding sites in *C. glabrata*. However, although there is no sequence similarity with known ScRpn4 binding motifs, *CgRPN4* gene expression was able to complement the absence of its *S. cerevisiae* homolog, *ScRPN4*, in BY4741_*Δrpn4* cells, suggesting that RPN4 binding site cannot be completely different in both organisms.

DISCUSSION

In this study, the first phenotypic screening on seventeen uncharacterized *C. glabrata* predicted TFs was undertaken in order to identify new players involved in pathogenesis related phenotypes. The seventeen transcription factors were selected for

their similarity to *C. albicans* transcription factors involved in multidrug resistance and biofilm formation, or their similarity to *S. cerevisiae* transcription factors involved in multidrug resistance and oxidative stress response. This analysis led to functional characterization of the *C. glabrata* CgRpn4 transcription factor.

CgRpn4 was demonstrated to play an important role in *C. glabrata* biofilm production under less nutritious conditions (SDB medium). This might occur due to the fact that when nutrients are exhausted, yeast cells enter stationary phase during which the synthesis of most proteins is attenuated and proliferation ceases. While biosynthetic processes are generally downregulated in stationary phase, prior studies have pointed to enhanced protein degradation during adaptation to nutrient deprivation in yeast [25]. Components of the ubiquitin-proteasome pathway are induced during this stage in order to degrade unneeded or damaged proteins. In fact, it was previously demonstrated that the impairment of proteasomal activity (using tea polyphenols) contributes to cellular metabolic and structural disruptions that expedite the inhibition of biofilm formation and maintenance by *C. albicans* [26]. However, relation with the growth media has not yet been reported. Therefore, since *CgRPN4* is a putative transcription factor for proteasomal genes, its absence in *C. glabrata* cells grown under less nutritious conditions impairs the proteasome pathway crucial for starving cells survival. Consequently, biofilm formation is probably also affected.

CgYAP1 was shown to be important for biofilm formation/maintenance in both rich and less nutritious medium. Several transcription factors involved in oxygen homeostasis and oxidative stress responses are targeted by *CgYAP1*, including *CgRPN4* [27]. This might suggest that, under less nutritious conditions, *CgRPN4* is the main target of *CgYAP1*. Reinforcing this idea, the biofilm production of *Δcgrpn4* and *Δcgyap1* mutants was found to be similar in SDB medium. Interestingly, *Δcgskn7* and *Δcgcad1* mutants also displayed great decrease in biofilm production. *CgYAP1*, *CgSKN7* and *CgCAD1* are involved in *C. glabrata* oxidative stress response and it was previously demonstrated that the elevated expression of anti-oxidant biomarkers is a likely cause of antifungal drug resistance commonly observed in biofilm mode *Candida* [28]. Additionally, it was demonstrated that *Candida* biofilms express a greater degree of antioxidant activity and contain significantly low ROS than their planktonic counterparts, meaning that anti-oxidants could contribute to the higher resistance to antifungals observed in *Candida* biofilms [28]. However, the importance of oxidative stress response-associated transcription factors in *C. glabrata* biofilm formation had not been elucidated until now. *CgHAP1* and *CgMRR1* were also shown to be involved in biofilm production. Although neither *CgMRR1* and *CgHAP1* or their *C. albicans* closest homologue *MRR1* have been reported to be induced in biofilm producing cells, *CgYAP1* was found to regulate these ORFs expression as well as *CgSKN7* and *CgRPN4* (Fig. 5). These findings suggest, for the first time, that *CgYAP1*, the major known regulator of oxidative stress resistance in *C. glabrata*, plays an important role in *C. glabrata* biofilm formation and regulates other predicted transcription factors that demonstrated to be involved in this process as well.

Concerning susceptibility assays, on one hand, none of the *C. glabrata* predicted TFs demonstrated to be involved in the resistance towards amphotericin B or flucytosine. On the other hand, the absence of *CgMRR1* and specially *CgRPN4* and *CgPDR1* was found to increase *C. glabrata* susceptibility towards azole antifungals. Previous studies reported that *C. glabrata* resistance toward azoles is largely mediated by the transcription factor *CgPDR1*, homologue of *S. cerevisiae PDR1*, resulting in the upregulation of ABC transporter proteins and drug efflux [23]. Single point mutations in *CgPDR1* have previously been shown to play a role in azole resistance development in clinical isolates [23]. In *S. cerevisiae*, Rpn4 is a transcription factor that stimulates expression of proteasome genes and is upregulated in strains harboring gain-of-function alleles of *PDR1* [23], and the same was reported to happen in *C. glabrata* by Caudle *et al.* (2011) [29]. These findings suggest that the proteasome may be influenced by *CgPDR1* via *CgRPN4* expression; however, the eventual role of *CgRPN4* in *C. glabrata* azole resistance had not yet been elucidated. Herein, CgRpn4 was shown to play an important role in *C. glabrata* resistance toward azole drugs and also against the echinocandin caspofungin. Nonetheless, the absence of *CgPDR1* was shown to exert a more detrimental effect on *C. glabrata* resistance to azoles than the absence of *CgRPN4*, suggesting that *CgPDR1* plays a major role in azole resistance, eventually in part through the regulation of *CgRPN4*. Using Pathoyeastract (http://www.pathoyeastract.org/), the interaction of CgPdr1p with *CgRPN4* was verified (Fig. 5). These results suggest that cross-regulation of *PDR1* and *RPN4* is conserved from *S. cerevisiae* to *C. glabrata*, with *CgPDR1* being the major azole drug-resistance regulator and *CgRPN4* an important azole-resistance player regulated by *CgPDR1*.

CgMRR1, *C. glabrata* closest homolog to *C. albicans* multidrug resistance regulator TF *MRR1*, demonstrated to be involved as well in azole-resistance, since *Δcgmrr1* mutant cells displayed growth impairment in the presence of inhibitory azoles. In *C. albicans*, *MRR1* controls the expression of *MDR1*, a MFS-MDR gene involved specifically in resistance to fluconazole rather than other azoles and is upregulated in drug-resistant clinical isolates. Interestingly, *CgMRR1* was found to be also regulated by the major azole drug-resistance regulator *CgPDR1* (Fig. 5). Altogether, the screening results highlight the multifactorial nature of the drug resistance and biofilm formation control in *C. glabrata*. Nonetheless, *CgRPN4* demonstrated to be an important player in both drug resistance and biofilm formation in *C. glabrata*, two process that contributes to the high prevalence of this human pathogen. Therefore, to further assess the role of this predicted transcription factor in the control of *C. glabrata* drug resistance, its functional analysis was undertaken. The overexpression of *CgRPN4* was found to greatly increase *C. glabrata* resistance to fluconazole and ketoconazole. Moreover, *CgRPN4* gene expression was able to complement the absence of its *S. cerevisiae* homolog (*ScRPN4*) in BY4741_*Δrpn4* mutant cells, increasing those cells resistance toward fluconazole and ketoconazole, strongly suggesting that *ScRPN4* and *CgRPN4* are orthologous genes. Interestingly, the overexpression of *CgRPN4* in wild-type *S. cerevisiae* BY4741 cells demonstrated to has a detrimental effect on this organism resistance. BY4741 cells harboring the cloning vector demonstrated lower susceptibility toward inhibitory concentrations of both fluconazole and ketoconazole than those overexpressing *CgRPN4*. Altogether these results suggest, on one hand, that *ScRPN4* and *CgRPN4* are orthologous genes, since the presence of these transcription factors was shown to be crucial for *S. cerevisiae* and *C. glabrata* cells survival, respectively, resistance toward azole antifungals. On the other hand, interestingly and unexpectedly, the overexpression of *CgRPN4* in BY4741 *S. cerevisiae* wild-type cells does not seem to bring any advantage concerning this organism drug resistance. Actually, BY4741 cells harboring the cloning vector demonstrated a slightly lower susceptibility toward inhibitory concentrations of both fluconazole and ketoconazole when compared to those overexpressing *CgRPN4*, through the recombinant plasmid. These results suggest that, in *S. cerevisiae*, the overexpression of Rpn4 has no positive effect in this organism resistance toward antifungals, as it has in *C. glabrata*. In fact, Wang *et al.* (2010) [30] demonstrated that inhibition of Rpn4p degradation dramatically sensitizes the cells to several genotoxic and proteotoxic stressors. These authors have shown that over-induction of Rpn4 is toxic if the protein cannot be removed rapidly by the proteasome. In this sense, a hypothesis is that CgRpn4 might not be controlled/degraded in *S. cerevisiae* in the same way as ScRpn4, leading to the apparent contradictory phenotypes observed. It was previously shown that degradation of

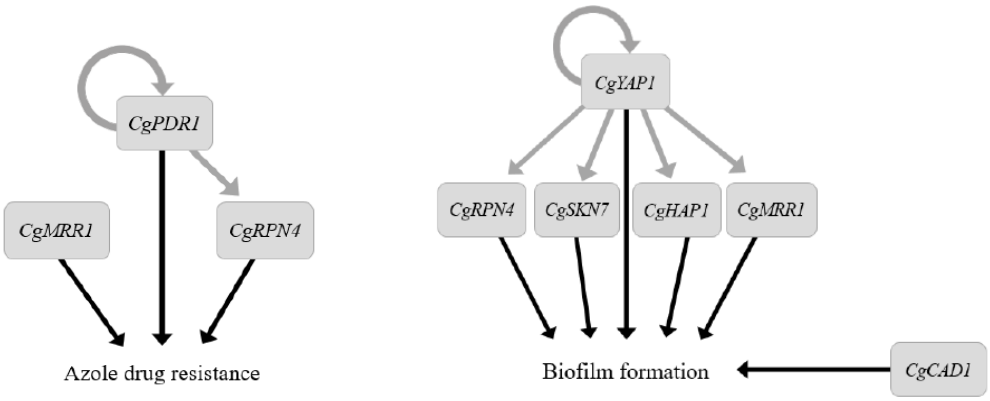

Figure 5 | Transcriptional regulation of drug resistance and biofilm formation in *C. glabrata*. Relation between the predicted transcription factors were assessed through Pathoyeastract (http://www.pathoyeastract.org/). Associated phenotype based on the results obtained in this study.

ScRpn4 can be both ubiquitin-dependent and -independent [31]. The portable Ub-independent degron is located from 1 to 80 amino acid residues, and portable Ub-dependent degron of was mapped from 172 to 229 residues. Comparing *ScRPN4* and *CgRPN4* amino acid sequences, it is clear that these two domains are poorly conserved between these homologs proteins, suggesting that CgRpn4 degradation in *S. cerevisiae* might not occur efficiently thus explaining the results obtained herein. On the other hand, *CgRPN4* overexpression levels are likely to be different in *S. cerevisiae* and *C. glabrata*, since different promoters were used to drive it. This difference in CgRpn4 expression may also justify the different effects of CgRpn4 overexpression in *C. glabrata* and *S. cerevisiae*. On the other hand, the possibility that Rpn4 target genes might not be exactly the same in both organisms, since the overexpressing of Rpn4 in *C. glabrata* constitutes an advantage concerning this pathogen survival under stress conditions whereas in *S. cerevisiae* its overexpression slightly impair cell growth under stress conditions, cannot be excluded.

To address the issue of CgRpn4 activation mechanisms, subcellular localization of CgRpn4p was assessed in different environmental conditions. Nuclear localization of the transcription factor was verified in both cells under control conditions and cells undergoing fluconazole or ketoconazole exposure. However, in exponential growing cells, nuclear localization was much less pronounced than in stationary phase cells. Actually, in *S. cerevisiae*, proteasome-dependent proteolysis was shown to be enhanced during early stationary phase and is then suppressed during late, or mature, stationary [32]. These results suggest that, although a large proportion of the population displays CgRpn4 accumulation in the nucleus even in unstressed exponential growth, there is a certain degree of increased nuclear accumulation of CgRpn4 under stress, both that induced by antifungal drugs or by reaching stationary phase of growth. Since CgRpn4 is a predicted transcription factor for proteasome genes, its target genes might be genes involved in cell survival at stationary phase, where increased oxidative stress and proteasome induction are verified [32]. When cells underwent azole drug exposure CgRpn4 concentrated even more in the cell nucleus, which suggest that ubiquitin-proteasome system may be controlled by transcriptional regulators of multidrug resistance via *RPN4* expression as it happens in closely related yeast *S. cerevisiae*. Additionally, besides proteasome-related genes, other genes related with multidrug resistance might be Rpn4 targets and susceptibility assays also suggest that. To further analyse this hypothesis, the effect of *CgRPN4* deletion in the transcriptome-wide response of *C. glabrata* cells toward mild fluconazole-induced stress conditions was assessed through RNA-sequencing.

Although the drug concentration used was not sufficiently inhibitory to induce a strong response in wild-type cells, the results obtained concerning the genes that are up or downregulated in both control and fluconazole-induced stress conditions, in the *Δcgrpn4* mutant compared to the wild-type strain, enabled the identification of a short list of genes whose expression was seen to depend on the CgRpn4 transcription factor. Additionally, the used replicates were found to be overall more different than expected, leading to a decreased number of genes whose expression differences is statistically significant. Interestingly, the *C. glabrata ScRPL29* homolog was found to be down-regulated via *CgRpn4* in cells under fluconazole-induced stress conditions, an observation that could partially explain the *Δcgrpn4* azole susceptibility phenotype, since *S. cerevisiae Δscrpl29* null mutant presents higher resistance toward miconazole [24].

Genes found to have significant expression changes in the *C. glabrata RPN4* deletion mutant relatively to the wild-type were grouped into predicted functional clusters. The majority of them were shown to be involved in RNA metabolism, in both control and fluconazole-induced stress conditions. Under stress conditions, several ribosomal biogenesis and translation- associated and protein trafficking- associated genes presented significant expression changes. In *S. cerevisiae*, it was previously shown that the repression of the ribosomal genes, along with a large set of genes involved in RNA metabolism, protein synthesis, and aspects of cell growth, is a general feature of the environmental stress response [33]. The inducible gene expression kinetics observed in response to stress is achieved by fine regulation of multiple steps of the mRNA biogenesis process. Although this is common to many stresses, the underlying mechanistic details of how such regulation is achieved are highly dependent on the particular stress and organism. Herein, all the four ribosomal biogenesis and translation-associated ORFs demonstrated to be downregulated by CgRpn4 under mild fluconazole-induced stress conditions. Nonetheless, concerning protein-trafficking and RNA metabolism-associated, both CgRpn4 up and downregulated ORFs were found.

Mitochondrial regulation-associated genes also demonstrated to be regulated by CgRpn4 in both control and fluconazole-induced stress conditions, being upregulated in its absence. In *S. cerevisiae*, mitochondrial respiratory functions were found to be overrepresented in H_2O_2 sensitive mutants [34]. Other studies demonstrated that, in *S. cerevisiae*, most of the energy generation gene products downregulated are mitochondrial electron transport proteins. It is speculated that these genes are downregulated in order to lessen the amount of endogenously generated reactive oxygen species (ROS) in the cell. This would be in response to generation of ROS due to the action of azoles on the cell membrane. Kobayashi and colleagues (2002) [35] demonstrated the production of ROS in *C. albicans* by miconazole and fluconazole, and there was a strong inverse correlation between the level of ROS production and the MIC. The authors hypothesize that resistant isolates may exhibit resistance mechanisms that involve scavenging ROS. On the other hand, François and colleagues (2006) [36] demonstrated that

fluconazole inhibits neither catalase nor peroxidase and does not induce a significant increase of ROS levels in *C. albicans*. The authors speculate that these differences might have occurred due to the different *C. albicans* strains used. Therefore, since *C. glabrata* is a different species, phylogenetically closer to *S. cerevisiae*, fluconazole-induced endogenous ROS level can be a hypothesis.

In previous studies, iron homeostasis genes were found to be downregulated in fluconazole-resistant clinical *C. albicans* isolates [37]. Here, two ORF products predicted to be involved in metal homeostasis were found to be upregulated in *Δcgrpn4* mutant, suggesting that in *C. glabrata CgRPN4* indirectly downregulates genes involved in metal homeostasis in response to fluconazole. Amino acid metabolism cluster were presented, under fluconazole-induced stress conditions, by a single CgRpn4 upregulated ORF homolog to *ScHIS3* which is involved in histidine biosynthesis. In fact, Rogers *et al.* (2002) [37] demonstrated that amino acid metabolism-associated genes were differently expressed in a *C. albicans* azole-resistant clinical isolate. However, there is no evidence connecting *C. glabrata* azole-resistance with amino acid metabolism so far. Moreover, several ORFs with still unknown function, with no *S. cerevisiae* or *C. albicans* homologs, demonstrated to be regulated by CgRpn4 in both control and fluconazole-induced stress conditions. Considering these novel observations, a model of *CgRPN4* regulation and action in *C. glabrata* under fluconazole exposure was hypothesized (Fig. 6).

Upstream regions of the ORFs found to be upregulated by CgRpn4 were analysed searching for a common motif that might correspond to the CgRpn4 binding site. The longest consensus sequence found in the predicted motifs that might correspond to a conserved part of known ScRpn4 binding motifs was CCAC. However, a 4 nucleotide length consensus sequence has a high random nucleotide distribution associated.

Within the 17 different binding motifs found to be enriched in CgRpn4 upregulated ORFs promoter regions, GAAGCA and AGTCTA were found in 5 out of the 6 inputted upstream regions, which suggest that these consensus sequences may be part of CgRpn4 binding sites in *C. glabrata*. However, although there is no sequence similarity with known ScRpn4 binding motifs, *CgRPN4* gene expression was able to complement the absence of its *S. cerevisiae* homolog, suggesting that Rpn4 binding site cannot be completely different in both organisms. Gasch *et al.* (2004) [38] explored the evolution of the proteasome *cis*-regulatory element in *S. cerevisiae* and *C. albicans*, particularly the case of *RPN4*, and noticed that, in addition to the Rpn4p consensus site, a number of related hexameric sequences were also highly enriched in the orthologous upstream regions from *C. albicans*, hinting the possibility that a different set of regulatory sequences control the expression of the *C. albicans* proteasome genes. They demonstrated that *S. cerevisiae* and *C. albicans* use different sequences to control the expression of the proteasome genes. Additionally, ScRpn4p and its orthologue CaRpn4p were shown to have different DNA-binding specificities. Unlike the evolutionary rates of protein coding regions, for which essential proteins typically evolve at a slower rate, the same authors found no evidence for a retarded rate of evolution/loss of the *cis*-regulatory systems of essential genes. For example, the proteasome subunits and the ribosomal proteins are among the most highly conserved proteins, and the genes that encode them are expressed with similar patterns in *S. cerevisiae* and *C. albicans*. Nevertheless, they identified different upstream sequences for these groups in the different species, suggesting that the regulation of the genes' expression has evolved even though their expression patterns have not. These observations might suggest that, even though *C. glabrata* is phylogenetically closer to *S. cerevisiae* than *C. albicans*, the regulation underlying orthologous genes expression may have diverged at consensus sequence level leading to differences in these genes regulatory regions. Interestingly, Ju *et al.* (2010) [39] demonstrated that, in *S. cerevisiae*, the nuclear localization signal of Rpn4 is located in the C-terminal half of Rpn4, from 206 to 531 residues, whereas the transactivation domain resides in the N-terminal region, from 11 to 210 residues. Comparing *ScRPN4* and *CgRPN4* amino acid sequences, it is clear that these two domains are poorly conserved among these yeasts. In contrast, comparing the C_2H_2 DNA binding domain of *ScRNP4*, from 477 to 507 amino acid residues [39], with the correspondent amionacid residues sequence in *CgRPN4* a high conservation level is verified. This observation supports the evidences showing that *CgRPN4* gene expression is able to complement the absence of its homolog *ScRPN4* in BY4741_*Δrpn4* cells. However, none of the ScRon4 known binding motifs were found in the upstream region of CgRpn4 upregulated ORFs.

Altogether, the results described in this study testify the importance of orthology relationships studies as an effort to unveil regulatory mechanisms in non-studied human pathogens. The

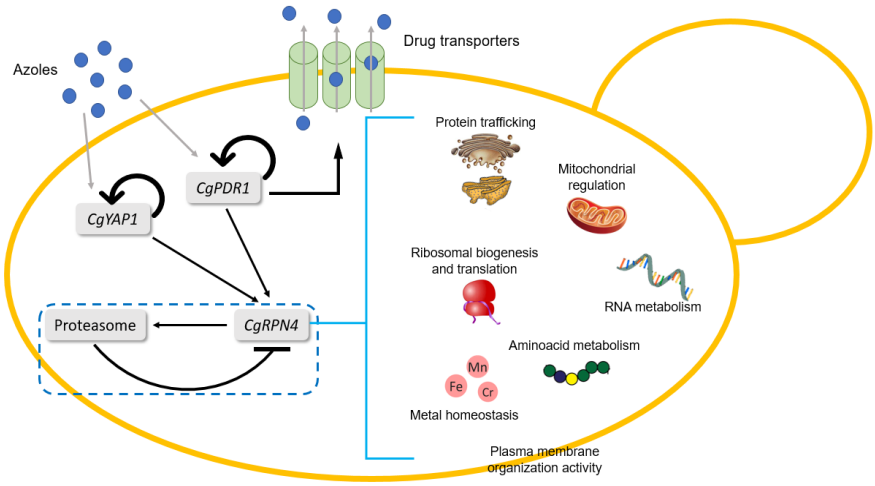

Figure 6 | Model of *CgRPN4* regulation and action in *C. glabrata* under fluconazole exposure. Azole drug exposure activates the pleiotropic drug resistance regulator *CgPDR1*, which besides regulates its own expression induces the expression of drug transporters such as ABC transporters (black arrows). Recently, it was demonstrated that *CgYAP1* also induce the expression of multidrug transporters, besides being the major known oxidative stress response regulator in *C. glabrata*. These two drug responsive genes, activates the expression of the transcription factor *CgRPN4* (black arrows) which activates proteasomal genes, such as in *S. cerevisiae*. In turn, the assembled proteasome degrades CgRpn4, in order to maintain a negative feedback loop (dark blue dashed line) to control proteome homeostasis and expression of Rpn4 target genes. Herein, a screening to analyse CgRpn4 targets in response to fluconazole was performed for the first time, through RNA-seq approach, unveiling several genes belonging to different functional clusters involved in different cellular processes being regulated by CgRpn4 (light blue bracket) in response to fluconazole. For instance, mitochondrial regulator, plasma membrane organization activity and metal homeostasis-associated genes were found to be downregulated by CgRpn4. This represents the first step to unveil the mechanism through which CgRpn4 expression influences *C. glabrata* resistance toward azole antifungals.

characterization of *CgRPN4* predicted transcription factor involved in proteasomal genes activation reinforce the need for study the mechanisms underlying the multidrug resistance phenomenon on *candida* species, specially *C. glabrata* due to its innate resistance to azole antimycotic therapy, as well as the mechanisms that lead to biofilm formation, which increases this human pathogen resistance to administrated antifungal drugs. This work also highlights the importance of genome/transcriptome-wide approaches in the study of possible resistance determinants like CgRpn4, as global approaches are very useful in identifying previously unforeseen or uncharacterized genes relevant for drug resistance phenotypes.

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