Contribution of *CgPDR1*-Regulated Genes in Enhanced Virulence of Azole-Resistant *Candida glabrata*

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**Abstract**

In *Candida glabrata*, the transcription factor CgPdr1 is involved in resistance to azole antifungals via upregulation of ATP binding cassette (ABC)-transporter genes including at least *CgCDR1*, *CgCDR2* and *CgSNQ2*. A high diversity of GOF (gain-of-function) mutations in *CgPDR1* exists for the upregulation of ABC-transporters. These mutations enhance *C. glabrata* virulence in animal models, thus indicating that *CgPDR1* might regulate the expression of yet unidentified virulence factors. We hypothesized that *CgPdr1*-dependent virulence factor(s) should be commonly regulated by all GOF mutations in *CgPDR1*. As deduced from transcript profiling with microarrays, a high number of genes (up to 385) were differentially regulated by a selected number (7) of GOF mutations expressed in the same genetic background. Surprisingly, the transcriptional profiles resulting from expression of GOF mutations showed minimal overlap in co-regulated genes. Only two genes, *CgCDR1* and *PUP1* (for PDR1 upregulated and encoding a mitochondrial protein), were commonly upregulated by all tested GOFs. While both genes mediated azole resistance, although to different extents, their deletions in an azole-resistant isolate led to a reduction of virulence and decreased tissue burden as compared to clinical parents. As expected from their role in *C. glabrata* virulence, the two genes were expressed as well in vitro and in vivo. The individual overexpression of these two genes in a *CgPDR1*-independent manner could partially restore phenotypes obtained in clinical isolates. These data therefore demonstrate that at least these two *CgPDR1*-dependent and -upregulated genes contribute to the enhanced virulence of *C. glabrata* that acquired azole resistance.

**Introduction**

*Candida glabrata* is a haploid member of Ascomycetes normally not found in the environment but which has rather adapted to conditions found in mammals [1]. Among human fungal pathogens, *C. glabrata* is often reported as the second most prevalent species after *Candida albicans* [2,3]. *C. glabrata* can cause mucosal and bloodstream infection (BSI) mainly in immunocompromised hosts. Worldwide, *C. glabrata* accounts for an average 11% of infections caused by *Candida* species, however this proportion varies from 7 to 20% depending on geographical locations [4].

*C. glabrata* infections can be treated with several antifungal agents including amphotericin B, azoles and echinocandins [5,6]. However, *C. glabrata* can develop antifungal resistance and especially to the class of azole antifungals. Azole resistance surveillance studies have revealed a proportion varying from 10 to 20% of isolates with MIC values reaching clinical breakpoints (e.g. 64 µg/ml for fluconazole, based on CLSI standards). Several countries reported an increase in the proportion of azole-resistant isolates from 2001 to 2007 [4]. *C. glabrata* is also known for exhibiting intrinsically higher azole MIC values than *C. albicans*. For example, the average of fluconazole MIC values of a *C. glabrata* wild type population is near a value of 4 µg/ml, while it is approximately 32-fold lower for *C. albicans* [7,8]. We and others showed that azole resistance in *C. glabrata* was mediated almost exclusively by enhanced drug efflux and overexpression of multidrug transporters of the ATP Binding Cassette (ABC) transporters. Several genes encoding these transporters were identified including *CgCDR1*, *CgCDR2* (PDH1) and *CgSNQ2* [8,9,10,11,12]. Azole resistance in clinical isolates can be the result of overexpression of single or several transporters [13]. The understanding of regulatory circuits controlling the expression of these genes has progressed in the recent years. A major regulator of these genes, *CgPDR1*, was identified [14,15]. This gene belongs to the family of zinc finger transcription factors and functionally resembles PDR1 and PDR3 from the baker’s yeast *Saccharomyces cerevisiae*. Deletion of *CgPDR1* results in a loss of transcriptional control of the major transporters involved in azole resistance and, consequently, decreased resistance to these antifungals [14,15]. *CgPDR1* exhibits mutations, so called gain-of-function (GOF) mutations, which are responsible for intrinsic high expression of ABC transporters and therefore constitute the molecular basis of azole resistance in *C. glabrata* [13,14,15]. One striking feature of GOF mutations is their high diversity among *CgPDR1* alleles from azole-resistant isolates. As many as 67 mutations conferring azole resistance are described up to now [13,14,15,16,17]. GOF mutations are found within several domains of the transcription
factor corresponding to putative functional elements inferred from comparison to the S. cerevisiae PDR1 and PDR3 and including the transcriptional activation domain, a regulatory domain and a so-called middle homology region (MHR) which is found in several zinc finger proteins [13,16].

Not only are GOF mutations in CgPDR1 important for azole resistance in C. glabrata but also for fungal-host interactions. We showed that GOF mutations were associated with enhanced virulence and fitness in animal models of systemic infection [13]. This was unexpected since it is generally accepted that the development of drug resistance in other microbes is usually associated with costs in virulence or fitness. Secondary compensatory mechanisms can however restore the costs of resistance development [18,19].

In this study we addressed in C. glabrata the identification of genes behind the GOF-dependent virulence of CgPDR1. Because we rationalized that some genes commonly expressed by GOF mutations could be responsible for this effect, we analysed with transcript profiling analysis C. glabrata isolates containing individual GOF mutations but in identical genetic backgrounds. Only two genes (CgCDR1 and PUP1) were identified. We describe here their relevance in the enhanced virulence mediated by CgPDR1 GOF mutations.

**Results**

**Transcriptional analysis of GOF mutations**

In a previous study, we reported a high variety of gain-of-function (GOF) mutations in the transcriptional activator CgPDR1 [13]. These mutations conferred azole resistance through the differentiated upregulation of several ABC transporters including CgCDR1, CgCDR2 and CgSNQ2. It is known that CgPDR1 controls the expression of many other genes, some of which contain a regulatory domain in their promoter matching the PDRF (Pleiotropic Drug Responsive Element) described in S. cerevisiae (TCCRYSR) [14,16].

We were therefore interested to test whether the differentiated expression pattern observed for a few genes as described earlier [13] could be generalized to the entire transcriptome of C. glabrata. In order to achieve this goal, labeled cRNA from mRNA isolated in triplicates from strains containing seven different CgPDR1 GOF mutants as compared to the wild type. Comparison to the factor corresponding to putative functional elements inferred from linear regression coefficients, which can establish the extent of gene co-regulation by pairs of separate GOF. As summarized in Table 2, approximately half of $r^2$ values from pairwise comparisons were above 0.5 (from 0.54 to 0.87) and thus signified a moderate trend towards the co-regulation of the genes by these GOFs. The highest correlation ($r^2 = 0.87$) was observed between expression patterns of GOF D1082G (SFY103) with P822L (SFY116) (Fig. 1A, left side). One GOF (R376W) in SFY101 yielded systematically low $r^2$ values with all other GOFs (between 0.0003 and 0.058). Increasing the cut-off for differential regulation to $\geq$3-fold did not significantly change $r^2$ values (data not shown). The expression of genes obtained from GOF P822L (SFY116) and from R376W is shown to illustrate the low level of gene co-regulation between both isolates (Fig. 1A, right side). Taken together, these data support the concept that individual GOF result each in distinct transcription profiles even though the number of GOF analysed is probably only a portion of the entire mutation spectrum.

Given the diversity of transcriptional profiles provided by each GOF, the generated transcriptional data were clustered in a separate analysis in order to group sets of genes co-regulated by the different GOFs. Four separated groups were thus identified which were enriched in specific biological processes (Fig. 1B). It is noteworthy that genes from cluster 1 and 4 are enriched in processes related to amino acid metabolism, while others are enriched in signal transduction and protein metabolic processes.

We closely inspected the transcription profiles of two isolates, one carrying the GOF mutation D1082G (SFY103) and the other the mutation P822L (SFY116). This choice was based on the fact that these profiles show the highest correlation ($r^2 = 0.87$) and similar numbers of up- and downregulated genes, thus facilitating comparisons (Table 1 and 2). Between the two GOFs, 86 genes were co-regulated (32 upregulated and 54 downregulated) from the total of 626 genes regulated by at least one GOF. The number of GOF analysed is probably only a portion of the entire mutation spectrum.

**Table 1. Number of C. glabrata genes regulated by $\geq$2-fold in PDR1 GOF mutants as compared to the wild type.**

| Strain | CgPDR1 GOF mutation | Genes upregulated | Genes downregulated | Total |
|--------|---------------------|-------------------|---------------------|-------|
| SFY101 | R376W               | 27                | 46                  | 73    |
| SFY103 | D1082G              | 53                | 77                  | 130   |
| SFY105 | T588A               | 235               | 150                 | 385   |
| SFY109 | E1083Q              | 58                | 103                 | 161   |
| SFY111 | Y584C               | 197               | 132                 | 329   |
| SFY115 | L280F               | 67                | 132                 | 199   |
| SFY116 | P822L               | 71                | 89                  | 160   |

**Table 2. Correlation coefficients of transcriptional profiles.**

| GOF in CgPDR1 allele | L280F | R376W | Y584C | T588A | P822L | D1082G | E1083Q |
|----------------------|-------|-------|-------|-------|-------|--------|--------|
| L280F                | 0.016 | 0.016 | 0.016 | 0.016 | 0.016 | 0.016  | 0.016  |
| R376W                | 0.016 | 0.016 | 0.016 | 0.016 | 0.016 | 0.016  | 0.016  |
| Y584C                | 0.611 | 0.611 | 0.611 | 0.611 | 0.611 | 0.611  | 0.611  |
| T588A                | 0.310 | 0.310 | 0.310 | 0.310 | 0.310 | 0.310  | 0.310  |
| P822L                | 0.309 | 0.309 | 0.310 | 0.310 | 0.310 | 0.310  | 0.310  |
| D1082G               | 0.053 | 0.053 | 0.053 | 0.053 | 0.053 | 0.053  | 0.053  |
| E1083Q               | 0.003 | 0.003 | 0.003 | 0.003 | 0.003 | 0.003  | 0.003  |
upregulated genes in the SFY103 vs SFY116 comparison showed enrichment for xenobiotic transporter activity ($p = 3.7\times10^{-3}$), while the downregulated genes exhibited enrichment in amino acid (arginine, glutamine) biosynthesis processes ($p = 5.87\times10^{-7}$ to $2.97\times10^{-6}$). The inspection of conserved motifs in the promoters of upregulated genes yielded the consensus YCCACGGA (Figure S3), which closely resembled the PDRE recognition motif of $PDR1$ in $S.\text{cerevisiae}$ ($\text{TCC[AG][CT[G/C][A/G]}$) [20]. These data are therefore consistent with the role of $CgPDR1$ in the regulation of genes by the GOF mutations D1082G and P822L.

To determine whether the expression of genes differentially regulated by the GOF mutations was also affected by the absence of $PDR1$, we analysed the expression profile of the $pdr1A$ strain SFY92. A total of 247 genes were differentially regulated (≥2-fold) in strain SFY92 as compared to SFY114 (containing the $CgPDR1$ wild type allele). Analysis of the 99 downregulated genes showed that one third of these genes encode for proteins predicted to be localized in the mitochondria. Moreover, enrichment of specific biological processes (oxidation-reduction, ATP synthesis coupled to electron transport chain, cellular respiration) was observed (File S2).

Consistent with these observations is that $PDR1$ and $PDR3$ in $S.\text{cerevisiae}$ are known to participate into the mitochondria-nucleus signalling pathway [21], which may also be applied to $CgPDR1$. Finally, 121 genes were differentially regulated not only in absence of $PDR1$ but also in the presence of GOF mutations, indicating that these genes might represent the basal set of $PDR1$-dependent genes.

Virulence determinants in $C.\text{glabrata}$

We reported that GOF mutations analysed here by transcriptional profiling in $C.\text{glabrata}$ not only resulted in azole resistance but also in enhanced virulence and fitness in a mice model of infection [13]. We reasoned that enhanced virulence could be due to specific genes commonly regulated by all $CgPDR1$ GOFs, given that this phenotype was shared by all these mutations. Our current analysis revealed that no gene was commonly downregulated and only two genes were commonly upregulated by at least two-fold by all GOFs, i.e. $CgCDR1$, the well-known ABC-transporter involved in azole resistance, and the ORF CAGL0M12947g, which we named $PUP1$ (for $PDR1$ UPregulated gene) in the present study. This gene is highly similar to $YIL077c$, a gene encoding a protein...
of unknown function thought to be located in the mitochondria. We tested this hypothesis in *C. glabrata* by the expression of a GFP-tagged version of *PUP1* in the azole-resistant clinical isolate DSY565. As shown in Fig. 2, the GFP signal could be detected in DSY565. Moreover, Mitotracker Red staining (Panel C), which specifically reveals mitochondrial punctuate and tubular structures, co-localized with GFP signals of Pup1-GFP. These results therefore confirmed that *PUP1* encodes a mitochondrial protein. *CgCDR1* and *PUP1* are overexpressed by all GOFs and therefore they may constitute good candidates to be responsible for the enhanced virulence observed in animal models. *In vitro*, both genes were dependent on the presence of *CgPDR1* (Fig. 3A). Moreover, *PUP1* contains two PDREs in its promoter (−770 to −763: TCCACCGGA; −740 to −733: TCCGTGGA) and *PUP1* expression was inducible by fluconazole (Fig. 3B) similarly to *CgCDR1*. Because they might be important for the enhanced virulence phenotype, these genes should also be expressed *in vivo*. We tested this hypothesis by injecting mice with strains expressing the GFP under the control of the *CgCDR1* promoter or fused to the *PUP1* ORF. Kidneys homogenates were recovered and analyzed by flow cytometry to identify GFP-positive yeast cells. As shown in Fig. 4, GFP could be easily detected in the azole-resistant background DSY565 (SFY168) that expresses GFP under the control of the *CgCDR1* promoter. This was not the case in the DSY562 background (SFY167), where GFP expression driven by the *CgCDR1* promoter is low. Similarly, GFP signals in yeast cells expressing the GFP-tagged *PUP1* were detectable in the DSY565 background (SFY174), but not in the DSY562 background (SFY175). The results are consistent with the *in vivo* experiments performed with both GFP-tagged genes and thus indicate that *CgCDR1* and *PUP1* are overexpressed by *CgPDR1* GOF both *in vitro* and *in vivo*. To test whether *CgCDR1* and *PUP1* were involved in *C. glabrata* virulence, mutants were constructed in both the genetic backgrounds of DSY562 and DSY565 resulting in strains SFY148 and SFY149 (*CgCDR1* mutants) and SFY150 and SFY151 (*PUP1* mutants), respectively. The deletion of the genes was verified by Southern analysis (see Figure S2). The constructed mutants were next injected intravenously in mice and mice survival was recorded over time. In this model, mice are immuno-compromised by cyclophosphamide treatment. In general, deletion of *CgCDR1* and *PUP1* in DSY562 background had no significant effects as compared to the azole-susceptible isolate DSY562 (Fig. 5). On the contrary, the deletion of *CgCDR1* or *PUP1* in DSY565 resulted in a significant decrease in virulence as compared to the wild type (SFY149 vs DSY565: *p* = 0.04; SFY151 vs DSY565: *p* = 0.02). Deleting both genes from DSY565 (SFY170) had a no significant effect as compared to single mutants. In addition, revertant isolates, SFY160 and SFY162, restored *PUP1* and *CgCDR1* expression, respectively, and the phenotype of the wild type parent.

Tissue burdens were assessed at day 7 post infection and are shown in Fig. 6. In this model, mice are immunocompetent and the endpoint measurement is not mice survival but rather tissue colonization by the infection agent. CFU values were compared with each other. In isolates derived from DSY562, it is interesting to observe that the deletion of *PUP1*, even if it did not result in a decrease of mice survival as compared to the wild type, significantly decreased kidney colonization. This decrease was compensated by the reintroduction of *PUP1* in the mutant (SFY160). This decrease was even more pronounced in the absence of both *PUP1* and *CgCDR1* (SFY169). In isolates derived from DSY565, the individual deletion of *CgCDR1* and *PUP1* (SFY150 and SFY151) decreased CFU counts in a significant
manner as compared to the parent strain, a change which was restored by revertants of the corresponding genes. The double deletion of PUP1 and CgCDR1 decreased CFU counts in comparison to all other conditions, as observed from DSY5652-derived strains, indicating that CgCDR1 and PUP1 deletions have an additive effect on tissue colonization.

Taken together, these results strongly suggest that CgCDR1 and PUP1, two genes upregulated by all CgPDR1 GOF mutations, are important for the enhanced virulence phenotype observed in theazole-resistant isolate DSY565. Decreased virulence from DSY565-derived strains was associated with decreased tissue colonization and mutant phenotypes could be reverted by the corresponding wild type genes.

Overexpression of CgCDR1 and PUP1 in a CgPDR1-independent manner

The overexpression of CgCDR1 and PUP1 is under the control of CgPDR1 in C. glabrata. We showed in the above experiments that both CgCDR1 and PUP1 have impact on C. glabrata virulence. However, these experiments were carried out in the background of a functional CgPDR1 and it is possible that other CgPDR1-dependent factors contribute to enhanced virulence of azole-resistant isolates. We therefore expressed CgCDR1 and PUP1 with a strong constitutive promoter (TDH3) in the background of a CgPDR1 deletion strain to avoid interference with such factors. As observed in Fig. 7, the engineered strains could overexpress both genes at different levels but still to higher levels than pdr1Δ
mutants. CgCDR1 levels were approximately equal to those measured in the azole-resistant isolate DSY565 (Fig. 7A), while PUP1 levels were higher (approx. 20-fold) when expressed under the control of the TDH3 promoter than the native promoter (Fig. 7B). However, both genes were expressed to similar levels in DSY562 and DSY565 as expected from the constitutive expression from the TDH3 promoter. Azole MICs strains were 32 µg/ml fluconazole in strains overexpressing CgCDR1 via the TDH3 promoter, while the fluconazole MICs were almost identical to the parent strains when PUP1 was overexpressed (1–2 µg/ml, Table 3), indicating that CgCDR1 is the major mediator of azole resistance in our strains.

The strains were next injected intravenously in mice and tissue burden were next assessed from kidneys and spleen from sacrificed animals (Fig. 8). In general, when CgCDR1 and PUP1 were overexpressed in a pdr1 Δ mutant background, tissue burdens were significantly increased as compared to the parent strains. The colonization was slightly lower when PUP1 was overexpressed as compared to CgCDR1.

When virulence of the same strains was tested in the immuno-suppressed mice model, the results showed no significant difference between strains overexpressing CgCDR1 or PUP1 as compared to the pdr1 Δ mutants (Fig. 9). A closer inspection of the obtained data still suggests that strains overexpressing CgCDR1 or PUP1 tended to be more virulent than their parents. At day 15 post-infection, 90% of the mice infected with the pdr1 Δ mutants survived, while approximately 70% survived when infected with the overexpressing strains (Fig. 9).

These results support the idea that the individual overexpression of CgCDR1 and PUP1 contributed moderately to virulence; however, their overexpression was more important for maintaining

Figure 4. Expression of CgCDR1 and PUP1 in vivo. Flow cytometry analysis of GFP-positive yeast cells was performed from mice kidneys. Groups of 4 mice were injected intravenously with 4×10⁷ CFU of C. glabrata strains. Mice were sacrificed at day 7 post-infection. Results are expressed as percents of GFP-positive events in FACS and represent values recorded separately for each mouse. Asterisks indicate statistically significant differences (*: P<0.05; **: P<0.01, ***: P<0.001). Strains SFY167 and SFY168 express the CgCDR1p-3xGFP construct and are derived from DSY562 and DSY565, respectively. Strains SFY173 and SFY174 express the PUP1-3xGFP construct and are derived from DSY562 and DSY565, respectively. As controls, kidneys of uninfected mice (mock) were analyzed alone or mixed with 1×10⁷ cells of SFY168 or SFY174 grown in YEPD. doi:10.1371/journal.pone.0017589.g004

Figure 5. Virulence of C. glabrata is dependent on CgCDR1 and PUP1. Survival curves of mice infected with DSY562 (panel A) and DSY565 (panel B) and derived mutants. Statistical differences were performed using the Log-rank Mantel-Cox test (Prism 5.0) by comparing survival curves of mice infected by the parental strains (DSY562 or DSY565) and by other strains as indicated. Asterisks indicate statistically significant differences (*: P<0.05; **: P<0.01, ***: P<0.001). NS indicates no significance (P>0.05). For strains derived from DSY562, the indicated names correspond to the following strains: pdr1 Δ: SFY92, cdr1 Δ: SFY148, CDR1 rev: SFY173, pup1 Δ: SFY150, PUP1 rev: SFY159, cdr1 Δ, pup1 Δ: SFY152. For strains derived from DSY565, the indicated names correspond to the following strains: pdr1 Δ: SFY94, cdr1 Δ: SFY149, CDR1 rev: SFY162, pup1 Δ: SFY151, PUP1 rev: SFY160, cdr1 Δ, pup1 Δ: SFY153. doi:10.1371/journal.pone.0017589.g005
Figure 6. \textit{C. glabrata} tissue burdens in murine infection models. Fungal tissue burdens in kidneys (panel A) and spleen (panel B) from BALB/c mice infected intravenously with $4 \times 10^7$ viable cells of \textit{C. glabrata} strains. Mice were sacrificed at day 7 post-infection. Results are expressed as CFUs per gram of tissue and represent values recorded separately for each of the ten mice. Geometric means are indicated by horizontal bars. Statistical comparisons are summarized above each panel. Asterisks indicate statistically significant differences (*: $P < 0.05$; **: $P < 0.01$, ***: $P < 0.001$). NS indicates no significance ($P > 0.05$). The symbol '-' indicates that the statistical comparison was not performed. Statistical differences were determined using the non-parametric Wilcoxon Rank sum tests (Prism 5.0). The origin of each strain is indicated; strain background (DSY562 and DSY565) is indicated by filled or empty symbols, respectively. See legend of Fig. 5 for strain designations.

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Our study revealed a larger set of genes regulated by at least one GOF compared to the susceptible parent. From the 109 genes regulated by at least two-fold in the resistant isolate, 34 were found regulated (out of 626 genes) in our study, among which \( \text{CgPDR1} \) and \( \text{PUP1} \), the latter being the most upregulated gene in their study. The differences in transcriptional profiles could be explained by several factors including experimental conditions, type of array technology and intrinsic difference between isolates used in all three studies. One major difference between our study and others is that we used an isogenic background in the reintroduction of the seven individual \( \text{CgPDR1} \) alleles, which prevents intrinsic strain variations. This is perhaps a reason for the difference between the number of genes regulated in at least one condition in our study (626 genes regulated by at least one GOF) and that of Tsai et al. [16] (45 genes regulated in at least one strain pair). This view is supported by separate results obtained with the transcriptional comparison of two related clinical strains, DSY717 and DSY2317, the latter containing the \( \text{CgPDR1} \) GOF L1081F. Between these two isolates, only 39 genes were regulated by at least two-fold (File S3), including \( \text{CgPDR1} \) and \( \text{PUP1} \), thus suggesting that intrinsic strain variations may mask the real effect of GOF on the \( C. \ glabrata \) transcriptome.

The overlap between our study and others [14,16] falls into 14 regulated genes (Figure S4). Besides \( \text{CgPDR1} \) and \( \text{PUP1} \), which were found consistently upregulated in all three studies, the other genes may constitute a core set of genes regulated by \( \text{CgPDR1} \). It is interesting to observe that the 14 genes are almost all found upregulated in the data provided by Vermitsky et al. [14] and Tsai et al. [16], while in our case, the regulation of these genes is dependent on the type of reintroduced GOF in the same genetic background. Several hypotheses will be provided below.

Given that \( \text{CgPDR1} \) is a major regulator of azole resistance in \( C. \ glabrata \) and should act on regulated genes via PDRE binding elements in the promoters of regulated genes, the consensus for \( \text{CgPDR1} \) binding (TCCRYGSR) was proposed and we searched systematically for this motif in the promoter regions of the 626

Figure 7. Overexpression of \( \text{CgCDR1} \) and \( \text{PUP1} \) in a \( \text{CgPDR1} \)-independent manner. Panel A: \( \text{TDH3} \)-dependent expression of \( \text{CgCDR1} \). Panel B: \( \text{TDH3} \)-dependent expression of \( \text{PUP1} \). Quantification was performed by qRT-PCR. The values are averages of three separate experiments and represent the increase in gene expression relative to SFY196 (set at 1.00). Strains derived from DSY562 are represented by black bars and the indicated names correspond to the following strains: \( \text{PDR1} \): SFY196, \( \text{pdr1A} \): SFY198, \( \text{pdr1A}+\text{TDH3p-CDR1} \): SFY200, \( \text{pdr1A}+\text{TDH3p-PUP1} \): SFY202. Strains derived from DSY565 are represented by white bars and the indicated names correspond to the following strains: \( \text{PDR1} \): SFY197, \( \text{pdr1A} \): SFY199, \( \text{pdr1A}+\text{TDH3p-CDR1} \): SFY201, \( \text{pdr1A}+\text{TDH3p-PUP1} \): SFY203. doi:10.1371/journal.pone.0017589.g007
genes regulated by at least one GOF in our study. Forty six (46) genes contained the consensus. We asked whether the degree of upregulation obtained by each GOF could be associated by the presence of the consensus. Our data show that the PDRE consensus was present in seven (for SFY101) to 45% (for SFY115) of the upregulated genes in single to several copies (see File S1). The presence of the PDRE could be detected in the downregulated genes, however the proportion was low (between 1–4%) and usually the detected PDRE occurred in a single copy. Regulatory elements on genes dependent on individual GOF were also searched with the RSAT tool (File S4). The following consensus elements on genes dependent on individual GOF were also searched with the RSAT tool (File S4). The following consensus site (TCCACCGA) could be detected in the promoters of upregulated genes from the GOF L280F (SFY115) and P822L (SFY116) and D1082G (SFY103). It resembles the PDRE consensus proposed by Vermitsky et al. [14] and fits to the sequence TCCACCGA published by Tsi et al. [16]. In complement to these analyses, we also observed that the PDRE consensus was present in 11 out of 14 promoters of regulated genes from three different data sets (Figure S4) and thus highlights the relevance of this binding site for the regulation of these genes. Future studies will be needed to address the genome-wide occupancy of CgPdr1 by chromatin immuno-precipitation experiments in C. glabrata. One can expect that CgPdr1 will bind to some extent to the genes commonly regulated by the different studies discussed here.

We showed here that GOF mutations in CgPDR1 have differential effect on transcriptional profiles. This result was unexpected since previous results investigating the effect of GOF mutations in regulators of drug resistance in other yeast species (for example MRR1 or TAC1 mutations in C. albicans) have concluded to a convergence of transcriptional profiles with different mutations on a same regulator [22,23,24]. As mentioned from data shown in Fig. 1A, while a pairwise comparison between two GOFs can yield good correlation between expressed genes, another example between R376W and P822L gave striking different results: here, about 55% of the regulated genes showed an inverse expression pattern. Such patterns is not unique to our study: Tsi et al. [16] have analysed the expression of a few genes including CgCDR1, CgPDR1, CgSNQ2 in a set of isogenic strains into which individual GOF were re-introduced. The authors observed a GOF-dependent gene expression pattern as documented here. Presently, no clear explanations could be provided four our observations. However, taking S. cerevisiae homologues Pdr1 and Pdr3 as models, some hypothesis can be formulated. In S. cerevisiae, the expression of the ABC-transporters PDR5, SNQ2, PDR10, PDR15 and YOR1 is controlled by Pdr1p/Pdr3p. In addition, Yrr1p modulates the expression of both SNQ2 and YOR1. Similarly to PDR3, YOR1 is autoregulated via PDREs in its promoter [25,26]. Pdr1p and Pdr3p can act as homo- or heterodimers and can positively or negatively regulate expression of target genes, indicating that additional factors can modulate their activity [27,28]. For instance, the transcriptional regulator Rdr1p, acts as a repressor of PDR5 in a PDRE-dependent manner and heterodimers of Rdr1p/Pdr1p or Rdr1p/Pdr3p compete with Pdr1p/Pdr3p for binding to PDREs [29,30]. Similarly, the zinc cluster protein Stb5p also acts through PDREs and forms predominantly heterodimers with Pdr1p (no interaction with Pdr3p or Yrr1p yet described). Yrr1p is only present as a homodimer [31]. Pdr1 and Pdr3 can also associate to different subunits of the Mediator complex including Med15 and Med12, which is an important step into the recruitment of RNA polymerase II for target gene transcription. These two subunits are present in the C- and L-Mediator complexes, which may act as positive and negative regulator of transcription, respectively [32]. While both Pdr1 and Pdr3 can bind to Med15, Pdr3 binds in a specific manner to Med12 only in cells with mitochondrial dysfunctions [32]. With respect to CgPdr1, which combined in a single gene properties shared by Pdr1 and Pdr3, these studies suggest that CgPdr1 may interact with other DNA-binding proteins and may also associate with different subunits of the Mediator complex. The different GOF detected in CgPdr1 may alter in a positive or negative manner these interactions and thus could result in differentiated gene expression patterns as observed in our study. Future studies will be needed to verify this hypothesis.

Virulence and tissue burden quantitative assays performed in this study support the idea that CgCDR1 and PUP1 are important for the pathogenesis of C. glabrata at some stage of the infection. Currently our data cannot discriminate whether or not C. glabrata can replicate in the tested animal models. At least, the tested strains can persist over the time course of the experimentation, which is consistent with similar experiments performed in mice [33]. Interestingly, enhanced virulence has been observed in other C. glabrata isolates where azole resistance results from mitochondrial dysfunctions independently of GOF CgPDR1 mutations. In this case, CgCDR1 and PUP1 are strongly upregulated and thus may also contribute to favor C. glabrata in host interactions [34]. The specific role of individual gene in fungal-host interaction remains to be solved however several reports have already identified ABC-transporters as able to contribute to selective advantages under host conditions. For example, the Cryptococcus neoformans ABC transporter AFR1 was shown to interfere with lysosome acidification in macrophages to increase its survival. In particular, azole-resistant isolates showing increased AFR1 expression were more virulent than their parental azole-susceptible isolates [35,36,37], which highlights the relevance of the association between drug resistance and virulence observed here. Interestingly, a recent study reported that AFR1 upregulation could be obtained by reversible chromosome duplication and thus suggests C. neoformans could use this mechanism to modulate its virulence [38]. In another fungal species, Botrytis cinerea, which is a fungus causing losses of commercially important fruits, vegetables and vineyards worldwide, ABC-transporter upregulation was
associated with drug resistance due to the use of fungicides. *B. cinerea* drug resistance is spreading, thus arguing against a fitness cost due to ABC-transporter upregulation [39]. Regarding *PUP1*, no other homologues were found yet involved in microbial pathogenesis and therefore the exact role of the product encoded by this gene in *C. glabrata* pathogenesis remains an open question.

We have attempted the overexpression of both genes in a *CgPDR1*-independent manner and animal experiments yielded results in favor of the hypothesis that *CgCDR1* and *PUP1* contribute to virulence. However, while tissue burden of mice were consistently increased when *CgCDR1* and *PUP1* were overexpressed (Fig. 8), virulence assays failed to discriminate in a...
statistical manner survival curves obtained with the overexpressing strains (Fig. 9). Several hypotheses could be provided explaining these results. First, it is possible that enhanced virulence needs the simultaneous overexpression of CgCDR1 and PUP1 to result in significant survival differences with parental strains. Second, it is also possible that, because the overexpression was carried out in a pdr1Δ mutant, other CgPDR1-dependent genes still need to be co-expressed for phenocopying the enhanced virulence of the original strain DSY565. Moreover, it is possible that the animal model used here (mouse intravenous infection) is not best suited to reveal the role of the two investigated genes. Urinary tract infection models might represent an alternative, as demonstrated by Domergue et al. [40]. These questions are currently being addressed in the laboratory.

In conclusion, our study started from a transcriptional analysis to identify important mediators of azole resistance and virulence in C. glabrata. The ABC transporter CgCDR1 contributes almost solely to azole resistance but has other activities contributing to the enhanced virulence of azole-resistant isolates. Nevertheless, this protein could be targeted for the design of inhibitors interfering both with resistance and virulence of this yeast species. ABC-transporter inhibitors have already been described and among them some are used in animal health for parasite protection (i.e. mylbecyins) and have low toxicity profiles for mammalian cells [41]. It will be therefore interesting to test these substances in the future to decrease drug resistance and its associated virulence in C. glabrata.

Materials and Methods

Strains and growth media

C. glabrata strains used in this study are listed in Table 4. Yeasts were grown in complete medium YEPD (1% Bacto peptone, Difco Laboratories, Basel, Switzerland), 0.5% Yeast extract (Difco) and 2% glucose (Fhuka, Buchs, Switzerland). To prepare inocula for experimental infections, yeasts were grown in YEPD medium. When grown on solid media, 2% agar (Difco) was added. YPD agar plates containing nourseothricin (clonNAT, Werner BioAgents) at 200 mg ml\(^{-1}\) were used as a selective medium for growth of yeast transformant strains. FLP-mediated excision of the ΔT1 cassette was induced by growing the cells for 4 h at 30°C in YCB-BSA medium (23.4 g l\(^{-1}\) yeast carbon base and 4 g l\(^{-1}\) bovine serum albumin; pH 4.0). One hundred to 200 cells were then spread on YPD plates containing nourseothricin (15 μg ml\(^{-1}\)) and grown for 48 h at 30°C to obtain nourseothricin-sensitive strains. This drug concentration can distinguish between nourseothricin-resistant and nourseothricin-sensitive cells. Escherichia coli DH5α was grown in Luria-Bertani broth or on Luria-Bertani agar plates supplemented with ampicillin (0.1 mg ml\(^{-1}\)) when required.

Drug susceptibility assays

The C. glabrata strains were tested for azole susceptibility with the broth microdilution method described in the EUCAST document EDef 7.1 [42]. Briefly, aliquots of 1.5×10\(^5\) cells ml\(^{-1}\) were distributed into wells of a microtiter plate in RPMI 1640 containing 2% glucose and incubated at 35°C for 24 h. Endpoint readings were recorded with an automatic plate reader (Multiskan Ascent, Thermo) and the lowest azole concentration that reduced growth to 50% of that of the drug-free control was defined as the MIC.

Construction of C. glabrata microarrays

The nucleotide sequences of the 5283 C. glabrata ORFs and the mitochondrial genome were downloaded from the Génolevure Consortium (http://www.genolevures.org/). Following the Agilent eArray Design guidelines, two separate probe sets for each ORF were designed, each consisting of 60 base oligonucleotides. The probe selection was performed using the GE Probe Design Tool. Probes were filtered following their base composition and distribution, cross-hybridization potential and melting temperature to yield two probe sets representing each 5210 nuclear and 6 mitochondrial ORFs. These probes cover more than 98% of the nuclear genome and represent 6 out of the 8 mitochondrial protein-encoding genes. For quality control and normalization purposes, 103 probes were selected randomly and spotted 20 times throughout each array in addition to standard Agilent controls including spike controls for intra- and inter-array normalizations. C. glabrata custom arrays were manufactured in the 8×15 k format by Agilent Technologies.

cRNA synthesis, one-color labelling and C. glabrata arrays hybridization

Sample preparation was performed on three biological triplicates. Total RNA was extracted from log phase cultures in
Table 4. Strains used in this study.

| Strain | Parental strain | Genotype | Reference |
|--------|-----------------|----------|-----------|
| DSY562 | Related to DSY565 | Azole-susceptible clinical strain | [11] |
| DSY565 |  | Azole-resistant clinical strain | [11] |
| DSY717 | Related to DSY2317 | Azole-susceptible clinical strain | [13] |
| DSY2317 |  | Azole-resistant clinical strain | [13] |
| SFY92  | DSY562 | pdr1::SAT1-FLIP | [13] |
| SFY93  | SFY92 | pdr1::FRT | [13] |
| SFY94  | DSY565 | pdr1::SAT1-FLIP | [13] |
| SFY95  | SFY94 | pdr1::FRT | [13] |
| SFY101 | SFY93 | pdr1::PDR1R376W::SAT1 | [13] |
| SFY103 | SFY93 | pdr1::PDR1L280F::SAT1 | [13] |
| SFY105 | SFY93 | pdr1::PDR1T588A::SAT1 | [13] |
| SFY109 | SFY93 | pdr1::PDR1P822L::SAT1 | [13] |
| SFY111 | SFY93 | pdr1::PDR1D1082G::SAT1 | [13] |
| SFY114 | SFY93 | pdr1::PDR1::SAT1 | [13] |
| SFY115 | SFY93 | pdr1::PDR1::SAT1 | [13] |
| SFY116 | SFY93 | pdr1::PDR1::SAT1 | [13] |
| SFY118 | DSY562 | cdr1::SAFl-FLIP | This study |
| SFY119 | DSY565 | cdr1::SAT1-FLIP | This study |
| SFY120 | DSY562 | pup1::SAT1-FLIP | This study |
| SFY151 | DSY565 | pup1::SAT1-FLIP | This study |
| SFY152 | SFY148 | cdr1::FRT | This study |
| SFY153 | SFY149 | cdr1::FRT | This study |
| SFY154 | SFY150 | pup1::FRT | This study |
| SFY155 | SFY151 | pup1::FRT | This study |
| SFY159 | SFY154 | pup1::PUP1::SAT1 | This study |
| SFY160 | SFY155 | pup1::PUP1::SAT1 | This study |
| SFY161 | SFY152 | cdr1::CDR1::SAT1 | This study |
| SFY162 | SFY153 | cdr1::CDR1::SAT1 | This study |
| SFY167 | DSY562 | CDR1::[pSF109] | This study |
| SFY168 | DSY565 | CDR1::[pSF109] | This study |
| SFY169 | SFY152 | cdr1::FRT, pup1::SAT1 | This study |
| SFY170 | SFY153 | cdr1::FRT, pup1::SAT1 | This study |
| SFY173 | DSY562 | PUP1::[pSF113] | This study |
| SFY174 | DSY565 | PUP1::[pSF113] | This study |
| SFY196 | DSY562 | ScTDH3p::SAT1 | This study |
| SFY197 | DSY565 | ScTDH3p::SAT1 | This study |
| SFY198 | SFY93 | pdr1::FRT, ScTDH3p::SAT1 | This study |
| SFY199 | SFY95 | pdr1::FRT, ScTDH3p::SAT1 | This study |
| SFY200 | SFY93 | pdr1::FRT, ScTDH3p::CDR1::SAT1 | This study |
| SFY201 | SFY95 | pdr1::FRT, ScTDH3p::CDR1::SAT1 | This study |
| SFY202 | SFY93 | pdr1::FRT, ScTDH3p::PUP1::SAT1 | This study |
| SFY203 | SFY95 | pdr1::FRT, ScTDH3p::PUP1::SAT1 | This study |

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Microarrays data analysis

Slides were washed and processed according to the Agilent 60-mer Oligo Microarray Processing protocol and scanned on an Agilent microarray scanner G2565BA (Agilent Technologies). Data were extracted from the images with Feature Extraction (FE) software (Agilent Technologies). FE software flags outlier features, and detects and removes spatial gradients and local backgrounds. Data were normalized using a combined rank consistency filtering with LOWESS intensity normalization.

The gene expression values obtained from FE software were imported into GeneSpring 10.0.2 software (Agilent Technologies) for preprocessing and data analysis. For inter-array comparisons, a linear scaling of the data was performed using the 75th percentile signal value of all of non-control probes on the microarray to normalize one-color signal values. Probe sets with a signal intensity value below the 20th percentile were considered as absent and discarded from subsequent analysis. The expression of each gene was normalized by its median expression across all samples. Genes were included in the final data set if their expression changed by at least 2-fold between each strain expressing a CgPDR1 GOF allele and the strain SFY114 expressing the CgPDR1 wild type allele at least 2 independent experiments. Corrected p-value (<0.05) was chosen as the cut-off for significance. Validation of genes found regulated by microarray analysis was performed by qRT-PCR analysis (see below for technical details) on a set of nine different genes. In general, the correlation found between qRT-PCR and microarray data was excellent (see Figure S1). Microarray data have been uploaded to the NCBI GEO microarray repository. The GEO accession number for the C. glabrata Agilent array is GPL10713 and the accession numbers for the data are GSE23827, GSE23828 and GSE23829.

Use of bioinformatic tools

The analysis of consensus pattern on C. glabrata promoters (~800 to ~1) was performed using the Regulatory Sequence Analysis Tools (RSAT: http://rsat.ulb.ac.be/rsat/index.html) and implemented to the pattern discovery tool (oligo-analysis). The settings were those supplied by default by the tool provider. The position-specific scoring matrices (PSSM) consensus matrices were
Quantitative real-time RT-PCR (qRT-PCR)

Total RNA was extracted from log phase cultures with an RNase-free DNase treatment step as previously described. Expression of the CgCDR1, CgCDR2 and CgSNQ2 genes was quantitatively assessed with real-time RT-PCR in an i-Cycler Q system (Bio-Rad). All primers and probes were designed with Beacon Designer 2 (version 2.06) software (Premier Biosoft International) and synthesized by MWG Biotech (Ebersberg, Germany). qRT-PCR were carried out as previously described.

Disruption and replacement of CgCDR1

For the disruption of CgCDR1, the SAT1 flipping method was employed (Reuss et al., 2004). The complete CgCDR1 ORF flanked by 500 bp was amplified by PCR from genomic DNA of DSY562 using the primers CgCDR1-ApaI (5'–gcgcaaaCTGGCCGATccattgagcttc-3') and PUP-SacII (5'–gcgcaaaGGGCCCgagtgaccatactacatta-3') containing KpnI and SacII restriction sites, and inserted into pBluescript II SK(+) to yield pSF90. PUP1 deletion was created by PCR using the primers PUP-Apal (5'–gcgcaaaGGGCCCgagtgaccatactacatta-3') and PUP-SacII (5'–gcgcaaaCTGGCCGATccattgagcttc-3') and pSF90 as a template. The resulting PCR product was digested with Apal and SacII and ligated to a 4.7 kb Apal-SacII fragment containing the SAT1 reporter cassette from pSF1 (45) to yield pSF94. This plasmid was linearised by digestion with KpnI and SacI and transformed into DSY562 and DSY565 to obtain the PUP1 deletion strains SFY150 and SFY151, respectively.

Another PUP1 deletion cassette was constructed to obtain strains with deletion in both CgCDR1 and PUP1. As described above, pSF90 was amplified using the primers PUP-Apal and PUP-SacII. The SAT1 marker without the flipper system was amplified using the primers SAT1-Apal (5'–gcgcaaaGGGCCCgagtgaccatactacatta-3') and SAT1-SacII 5'–ataagaatCCGGCGGctgagcttgacaggtcga-3') and inserted into pBluescript II SK(+) to yield pSF101. This plasmid was transformed into the CgCDR1 deletion strain SFY148 and SFY149 to obtain the CgCDR1 and PUP1 double deletion strains SFY169 and SFY170, respectively.

For PUP1 replacement, the SAT1 cassette was excised in SFY150 and SFY151 to obtain the nourseothricin-sensitive strains SFY154 and SFY155 respectively. PUP1 replacement cassette was created by PCR using the primers PUP-Apalb (5'–gcgcaaaGGGCCCgagtgaccatactacatta-3') and PUP-SacIb (5'–gcgcaaaCCGGCGGctgagcttgacaggtcga-3') and pSF90 as a template. The resulting PCR product was digested with Apal and SacII and ligated to a 4.7 kb Apal-SacII fragment containing the SAT1 reporter cassette from pSF1 to yield pSF98. This plasmid was linearised by digestion with KpnI and SacI and transformed into SFY154 and SFY155 to obtain the PUP1 revertant strains SFY159 and SFY160. All above-constructed strains were verified by Southern blot analysis (see Figure S2). Transformants were selected onto nourseothricin-containing YEPE plates.

Overexpression of CgCDR1 and PUP1

For CgCDR1 and PUP1 overexpression, the SAT1 marker was amplified using the primers SAT1-NotI (5’-ataagaatCCGGCGGCtctgacaggtcga-3') and SAT1-BamHI (5’-gcgcaaaGGGCCCgagtgaccatactacatta-3') and inserted into the NotI-BamHI restriction sites of pBluescript II SK(+) to yield pSF30. This plasmid was then digested with XhoI and EcoRI and ligated to a 1.3 kb XhoI-EcoRI fragment containing the C. glabrata CEN-ARS from pCAGACU-5 (Kitada et al., 1996) to yield pSF126. The 0.7 kb EcoRI-BamHI fragment from yEpGAP-Cherry-MCS (46) containing the constitutive S. cerevisiae TDI3 promoter, was ligated into pSF126 to yield pSF127. The complete CgCDR1 and PUP1 ORFs were amplified by PCR from genomic DNA of DSY562 using the primers CgCDR1-EcoRIfor (5’–actGAATTCCtctgctgacaggtcga-3') and CgCDR1-EcoRIrev (5’-ataGAATTCCtctgctgacaggtcga-3') and
PUP-EctaRlrev (5′-ataGAATTCggagactattgtgtagcaagg-3′), respectively. The resulting PCR products were digested by EcoRI and inserted downstream of the TDIH promoter of pSF127 to yield the CgCDR1 and PUP1 overexpressing vectors, pSF129 and pSF130, respectively.

The plasmids pSF129 and pSF130 were transformed into the PDR1 deletion strains SYF93 and SYF95 to obtain strains overexpressing CgCDR1 (SYF200 and SYF201) or PUP1 (SYF202 and SYF203). As controls, plasmid pSF127 was introduced in strains DSY562, DSY565 and derivatives pbdA mutants SYF93 and SYF95 to yield strains SYF196, SYF197, SYF198 and SYF199, respectively. Transformants were selected onto nourseothricin-containing YEPD plates.

Construction of the fusions CgCDR1p-3xGFP and PUP1-3xGFP

To express GFP under the control of the CgCDR1 promoter, the S/II marker was amplified using the primers SAT1-Stul (5′-ataagaATGGCTgccaactaggaataaag-3′) and SAT1-BamHI (see above) and inserted into the Stul-BglII restriction sites of pBS-3xGFP–TRP1 [47] containing three tandemly fused GFP genes (3xGFP) to yield pSF104. Five hundred bp of the CgCDR1 promoter were amplified from genomic DNA of the plasmids CgCDR1p-BamHI (5′-gcgcaaaGGATCCtacatatgttcacacacag-3′) and CgCDR1p-BclI (5′-gcgcaaaATCCATgagatctgagctg-3′) containing BamHI and BglII restriction sites, respectively, and inserted into the BamHI site of pSF104 to yield pSF109. This plasmid was linearised by digestion with SphI and transformed into DSY562 and DSY563 to obtain strains SYF167 and SYF168, respectively.

To fuse the 3xGFP gene and the PUP1 ORF, the complete PUP1 ORF was amplified from DSY562 genomic DNA using the primers PUP-BglIII (5′-gcgcaaaAGATCTtagtgcagacag-3′) and PUP-BglII (5′-gcgcaaaAGATCTtagtgcagacag-3′) and inserted into the BamHI site of pSF104 to yield pSF113. This plasmid was linearised by digestion with NotI and transformed into DSY562 and DSY563 to obtain strains SYF173 and SYF174, respectively. Transformants were selected onto nourseothricin-containing YEPD plates.

Confocal microscopy

To label mitochondria, log phase cultures of strain SFY174 were treated with 0.25 μM Mitotracker® Red CMXRs (Molecular Probes) for 30 min and washed with PBS. C. glabrata cells were fixed in 3.5% para-formaldehyde at 4°C for 5 min following 10 min at room temperature. Cells were then washed 3–5 min with phosphate-buffered saline (10 mM Na2HPO4, 2 mM KH2PO4, 140 mM NaCl, 3 mM KCl, pH 7.4). The remaining fixative was washed and replaced with Tris-HCl, pH 8.0. Fluorescence was analyzed with a confocal fluorescence microscope (Zeiss LSM 510 Meta, Jena, Germany).

Flow cytometry

Groups of four female BALB/c mice (20 to 25 g; Charles-River) were injected into their lateral vein with saline suspensions containing 4×107 colony-forming units (CFU) of the C. glabrata strains (each in a volume of 250 μl). After seven days, mice were sacrificed by CO2 inhalation and kidneys were excised aseptically and homogenized in 10 ml sterile water. Kidneys homogenates were washed twice with FACS buffer (1×PBS pH 7.4, 5% FCS, 2 mM EDTA pH 8.0) and resuspended in 2 ml FACS buffer. Remaining tissue aggregates and cell clumps were eliminated by filtration through 50-μm cell strainers. A FACSCalibur® system (BD Bioscience) and the CellQuest™ software were used for analysis.

Animal studies

Female BALB/c mice (20 to 25 g) were purchased from Harlan Italy S.r.l (San Pietro al Natisone, Udine, Italy) and inbred in-house. The mice were housed in filter-top cages with free access to food and water. To establish C. glabrata infection, mice were injected into their lateral vein with saline suspensions of the C. glabrata strains (each in a volume of 200 μl).

In virulence studies, a group of ten immuno-suppressed mice was established for each yeast strain. Mice were rendered neutropenic by intraperitoneal administration of cyclophosphamide (200 mg kg−1 of body weight per day) three days before challenge and on the day of infection. Mice were injected with 7×107 colony-forming units (CFU) of each of the investigated strains. For tissue burden experiments, immuno-competent mice were inoculated with 4×107 CFU. After seven days, mice were sacrificed by use of CO2 inhalation, and target organs (spleen and kidney) were excised aseptically, weighed individually, and homogenized in sterile saline by using a Stomacher 80 device (Pbi International, Milan, Italy) for 120 s at high speed. Organ homogenates were diluted and plated onto YPD. Colonies were counted after two days of incubation at 30°C, and the numbers of CFU g−1 of organ were calculated. For survival experiments, mice were made neutropenic as previously described [48] and then injected with 7×107 CFUs of each of the strains studied. Mice were monitored with twice-daily inspections and those that appeared moribund or in pain were sacrificed by use of CO2 inhalation.

CFU counts were analyzed with non-parametric Wilcoxon Rank sum tests, while mean survival times were compared among groups by using the long-rank test. A P-value of less than 0.05 was considered to be significant.

Ethics Statement

The animal experiments were performed under a protocol approved by the Institutional Animal Use and Care Committee at Università Cattolica del S. Cuore, Rome, Italy (Permit number: L21, 10/02/2008) and authorized by the Italian Ministry of Health, according to Legislative Decree 116/92, which implemented the European Directive 86/609/EEC on laboratory animal protection in Italy. Animal welfare was routinely checked by veterinarians of the Service for Animal Welfare. Animal experiments carried out for in vivo detection of GFP-tagged proteins (see above) were performed at the University of Lausanne and University Hospital Center under the surveillance of the local governmental veterinarian offices. These experiments were approved by the local governmental veterinarian offices and are registered under number 1734.2.

Supporting Information

Figure S1 Validation of microarrays results by qRT-PCR. Panel A: Gene expression relative to the strain SFY114 (containing the wild type CgPDR1 allele) obtained by microarray analysis for each of the investigated GOF mutation in CgPDR1. Color code for up- and downregulated genes is given. Panel B: Gene expression relative to the strain SFY114 obtained by qRT-PCR. The values are averages of three separate experiments and represent increase in gene expression relative to SFY114 (set at 1.00). Primers used for CgPDR1, PUP1, CgCDR1 and the normalization control CgACT1 are described in the Material and Methods section. Other primers used for qRT-PCR are listed.
below. The comparison between qRT-PCR results and microarrays was estimated by linear regression between relative expression changes. R² values ranged from 0.4 and 0.09 between comparisons. Two comparisons including values obtained for CAGL0A00473g and CAGL0A00475g (PDR1) gave low correlation coefficients. This is explained by the fact that microarrays values of regulated genes were 10–100 fold different than observed for qRT-PCR. However, these discrepancies do not change the categorization of these genes being up- and downregulated by a given GOF mutation and taking a 2-fold change as a cut-off value. Forward and reverse primers are the following for CAGL0K00715g: 5'-TGCAACATCGAAGTGGTAAAG-3' and 5'-CATTCACATTTCCTGCGGTG-3'; for CAGL0A00473g: 5'-CACCTGGTGCGCTGAAAGGTG-3' and 5'-CGATCTCTCCCCTAGGGCAATC-3'; for CAGL0F09724g: 5'-GCCCTGAGACTGGTGACACC-3' and 5'-TTGTGGGACGTGTCTTCGA-3'; for CAGL0D0662g: 5'-CGCTGATGTTTCTGCGATG-3' and 5'-CAGCGGATGCATC-3'.

Figure S2 Southern blot analysis and diagram illustrating strategies for disruption and replacement of CgCDR1 and PUP1 in C. glabrata isolates. DNA was purified from isolated colonies, digested with the restriction enzyme PstI, analyzed by gel electrophoresis and hybridized to specific probes. **Panel A:** Analysis of CgCDR1 loci. The expected sizes for CgCDR1 analysis are: 1.7 kb for DSY562 and DSY565 (wild type CgCDR1 locus); 6.1 kb for SFY148 and SFY149 (cdr1Δ::XMT1-FLP); 1.3 kb for SFY152, SFY153, SFY169 and SFY170 (cdr1Δ::XRT); 1.7 kb for SFY161 and SFY162 (cdr1Δ::CgCDR1-SATT). **Panel B:** Analysis of PUP1 loci. The expected sizes for PUP1 analysis are: 1.2 kb for DSY562 and DSY565 (wild type PUP1 locus); 12.6 kb for SFY150 and SFY151 (pup1Δ::XMT1-FLP); 7.8 kb for SFY154 and SFY155 (pup1Δ::XRT); 1.2 kb for SFY159 and SFY160 (pup1Δ::PUP1-SATT); 9.7 kb for SFY169 and SFY170 (pup1Δ::SATT).

Figure S3 Promoter consensus analysis of genes upregulated in SFY103 (GOF mutation D1082G) and SFY116 (GOF mutation P822L). The data was obtained using RSAT (http://rsat.ulb.ac.be/rsat/index.html) and the oligo-analysis tool with default settings.

References

1. Kaur R, Donnerge R, Zupanic ML, Cormack BP (2005) A yeast by any other name: Candida albicans and its interaction with the host. Curr Opin Microbiol 8: 370–384.
2. Ruhnke M (2006) Epidemiology of Candida albicans infections and role of non-Candida albicans yeasts. Curr Drug Targets 7: 495–504.
3. Pfänder MA, Diekema DJ (2007) Epidemiology of invasive candidiasis: a persistent public health problem. Clin Microbiol Rev 20: 133–163.
4. Pfänder MA, Diekema DJ, Gibbons DL, Newell VA, Barton R, et al. (2010) Geographic variation in the frequency of isolation and fluconazole and voriconazole susceptibilities of Candida glabrata, an assessment from the ARTEMIS DISK Global Antifungal Surveillance Program. Diag Microbiol Infect Dis 67: 162–171.
5. Sanglard D, Odds FC (2002) Resistance of Candida species to antifungal agents: molecular mechanisms and clinical consequences. Lancet Infect Dis 2: 73–85.
6. Lass-Floët C (2009) The changing face of epidemiology of invasive fungal disease in Europe. Mycoses 52: 197–205.
7. Borst A, Rainer MT, Warmack DW, Morrison CJ, Ashington-Skaggs BA (2005) Rapid acquisition of stable azole resistance by Candida glabrata isolates obtained before the clinical introduction of fluconazole. Antimicrob Agents Chemother 49: 783–787.
8. Sanglard D, Ischer F, Bille J (2001) Role of ATG-binding-cassette transporter genes in high-frequency acquisition of resistance to azole antifungals in Candida glabrata. Antimicrob Agents Chemother 45: 1174–1183.
9. Torrelli R, Posterozo B, Ferrari S, De Carolis E, La Sorda M, et al. (2008) The ATG-binding cassette transporter-encoding gene CgVOR is a contributor to the CgPdr1-dependent azole resistance in Candida glabrata. Mol Microbiol 68: 186–201.
10. Bennett JE, Iszumaki K, Moe KA (2004) Mechanism of increased fluconazole resistance in Candida glabrata during prophylaxis. Antimicrob Agents Chemother 48: 1737–1743.
11. Sanglard D, Ischer F, Calabrèse D, Majcherzynki PA, Bille J (1999) The ATP-binding cassette transporter gene CgPDR1 from Candida glabrata is involved in the resistance of clinical isolates to azole antifungal agents. Antimicrob Agents Chemother 43: 2753–2754.
12. Iszumaki K, Kakeya H, Tsai HF, Grimberg B, Bennett JE (2003) Function of C. glabrata ABC transporter gene, PDR1. Yeast 20: 249–261.
13. Ferrari S, Ischer F, Calabrèse D, Posterozo B, Sanguinetti M, et al. (2009) Gain of function mutations in CgPDR1 of Candida glabrata not only mediate antifungal resistance but also enhance virulence. PLoS Pathog 5: e1000268.
14. Vermidys JP, Earhart KD, Smith WL, Honayoumi R, Edlin TD, et al. (2006) PDR1 regulates multidrug resistance in Candida glabrata: a genome disruption and genome-wide expression studies. Mol Microbiol 61: 704–722.
15. Tsai HF, Krol AA, Sarti KE, Bennett JE (2006) *Candida glabrata PDR1*, a transcriptional regulator of a pleiotropic drug resistance network, mediates azole resistance in clinical isolates and petite mutants. Antimicrob Agents Chemother 50: 1384–1392.

16. Tsai HF, Sammons LR, Zhang X, Saffi SD, Su Q, et al. (2010) Microarray and molecular analyses of the azole resistance mechanism in *Candida glabrata* oropharyngeal isolates. Antimicrob Agents Chemother 54: 3308–3317.

17. Berila N, Saleh A, Brandl CJ (1996) Transcriptional activation by yeast zinc cluster protein Rdr1p is a transcriptional repressor of the *Saccharomyces cerevisiae* gene encoding a multidrug transporter. J Biol Chem 271: 15814–15820.

18. Liu TT, Znaidi S, Barker KS, Xu L, Homayouni R, et al. (2007) Genome-wide expression and location analyses of the *Candida albicans* Tac1p regulon. Eukaryot Cell 6: 2122–2138.

19. Siano E, Lee H, Chang YC, Kwon-Chung KJ (2010) Heteroresistance to fluconazole in *Cryptococcus neoformans* is intrinsic and associated with virulence. Antimicrob Agents Chemother 53: 2094–2103.

20. Dryden MW, Payne PA (2005) Preventing parasites in cats. Vet Ther 6: 260–267.

21. Morschhauser J, Barker KS, Liu TT, Bla BWJ, Homayouni R, et al. (2007) Genome-wide analysis of the *Candida glabrata* MDRI gene reveals novel transcriptional regulation. J Biol Chem 282: 16110–16119.

22. Coste AT, Karababa M, Bille J, Sanglard D (2004) TAC1, a transcriptional activator of *Saccharomyces cerevisiae* CDR genes, is a new transcription factor involved in the regulation of *Candida albicans* ABC transporters *CDR1* and *CDR2*. Eukaryot Cell 3: 1639–1652.

23. Reuss O, Vik A, Kolter R, Morschhauser J (2004) The *Saccharomyces cerevisiae* ABC transporter-encoding gene *MDR1* affects the resistance of *Cryptococcus neoformans* to microglia-mediated antifungal activity by delaying phagosome maturation. FEMS Yeast Res 9: 301–310.

24. Tsai HF, Sammons LR, Zhang X, Suffis SD, Su Q, et al. (2010) Microarray and molecular analyses of the azole resistance mechanism in *Candida glabrata* oropharyngeal isolates. Antimicrob Agents Chemother 54: 3308–3317.

25. Martens JA, Genereaux J, Saleh A, Brandl CJ (1996) Transcriptional activation by yeast zinc cluster protein Rdr1p is inhibited by its association with NGG1p/ADA3p. J Biol Chem 271: 4359–4366.

26. Liu TT, Znaidi S, Barker KS, Xu L, Homayouni R, et al. (2007) Genome-wide expression and location analyses of the *Candida albicans* Tac1p regulon. Eukaryot Cell 6: 2122–2138.

27. Siano E, Chang YC, Garraffo HM, Kwon-Chung KJ (2009) Heteroresistance to fluconazole in *Cryptococcus neoformans* is intrinsic and associated with virulence. Antimicrob Agents Chemother 53: 2094–2103.

28. Coste AT, Karababa M, Bille J, Sanglard D (2004) TAC1, a transcriptional activator of *Saccharomyces cerevisiae* CDR genes, is a new transcription factor involved in the regulation of *Candida albicans* ABC transporters *CDR1* and *CDR2*. Eukaryot Cell 3: 1639–1652.

29. Akache B, Turcotte B (2002) New regulators of drug sensitivity in the family of yeast zinc finger regulators *Pdr1p and Pdr3p control pleiotropic drug resistance network, mediates multidrug resistance in *Saccharomyces cerevisiae*. J Biol Chem 277: 21254–21260.

30. Shahi P, Ghulian K, Naar AM, Moye-Rowley WS (2010) Differential Roles of Transcriptional Mediator Subunits in Regulation of Multidrug Resistance Gene Expression in *Saccharomyces cerevisiae*. Mol Biol Cell 21: 2469–2482.

31. Jacobson ID, Brunke S, Seiler K, Schwarzmüller T, Firon A, et al. (2010) *Candida glabrata* persistence in mice does not depend on host immunosuppression and is unaffected by fungal amino acid auxotrophy. Infection and Immunity 78: 1066–1077.

32. Tsai HF, Krol AA, Sarti KE, Bennett JE (2006) *Candida glabrata PDR1*, a transcriptional regulator of a pleiotropic drug resistance network, mediates azole resistance in clinical isolates and petite mutants. Antimicrob Agents Chemother 50: 1384–1392.

33. Shahi P, Ghulian K, Naar AM, Moye-Rowley WS (2010) Differential Roles of Transcriptional Mediator Subunits in Regulation of Multidrug Resistance Gene Expression in *Saccharomyces cerevisiae*. Mol Biol Cell 21: 2469–2482.

34. Jacobson ID, Brunke S, Seiler K, Schwarzmüller T, Firon A, et al. (2010) *Candida glabrata* persistence in mice does not depend on host immunosuppression and is unaffected by fungal amino acid auxotrophy. Infection and Immunity 78: 1066–1077.

35. Ferrari S, Sanguinetti M, De Bernardis F, Torelli R, Posteraro B, et al. (2011) Loss of mitochondrial functions associated with azole resistance in *Candida glabrata* also results in enhanced virulence in mice. Antimicrob Agent Chemother (in press).

36. Sanguinetti M, Posteraro B, La Sorda M, Torelli R, Fiori B, et al. (2006) Role of *APR1*, an ABC-transporter-encoding gene, in the in vivo response to fluconazole and virulence of *Cryptococcus neoformans*. Infect Immun 74: 1352–1359.

37. Orsi CF, Colomboari B, Ardizzone A, Peppoloni S, Neglia R, et al. (2009) The ABC transporter-encoding gene *APR1* affects the resistance of *Cryptococcus neoformans* to microglia-mediated antifungal activity by delaying phagosome maturation. FEMS Yeast Res 9: 301–310.

38. Sanguinetti M, Posteraro B, La Sorda M, Torelli R, Fiori B, et al. (2006) Role of *APR1*, an ABC-transporter-encoding gene, in the in vivo response to fluconazole and virulence of *Cryptococcus neoformans*. Infect Immun 74: 1352–1359.

39. Orsi CF, Colomboari B, Ardizzone A, Peppoloni S, Neglia R, et al. (2009) The ABC transporter-encoding gene *APR1* affects the resistance of *Cryptococcus neoformans* to microglia-mediated antifungal activity by delaying phagosome maturation. FEMS Yeast Res 9: 301–310.

40. Sanguinetti M, Posteraro B, La Sorda M, Torelli R, Fiori B, et al. (2006) Role of *APR1*, an ABC-transporter-encoding gene, in the in vivo response to fluconazole and virulence of *Cryptococcus neoformans*. Infect Immun 74: 1352–1359.