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Multiple interfaces control activity of the *Candida glabrata* Pdr1 transcription factor mediating azole drug resistance

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Abstract

The Cys₆Zn₂ DNA-binding domain transcription factor Pdr1 is a central regulator of drug resistance in the pathogenic yeast *Candida glabrata*. In this review, I discuss the multiple control mechanisms modulating the function of this positive transcriptional regulator. Available data suggest that Pdr1 activity is restrained by multiple negative inputs that can be lost by either mutagenesis of the protein or loss of trans-acting factors. Although extensive data are available on the *C. glabrata* transactivator as well as its cognate proteins in *Saccharomyces cerevisiae*, the physiological rationale underlying the regulation of these factors remains to be understood.

Introduction

Azole drugs are one of the most important classes of antifungal drugs available. While administration of other antifungal agents requires hospitalization, azole drugs can be delivered orally. This feature, along with their relative high tolerance, has made these antifungal drugs the most commonly utilized chemotherapeutics in the clinic.

Given the widespread use of azole drugs, resistance to these antifungal drugs is a major clinical complication in treatment of fungal disease. The most common fungal disease is caused by the *Candida* genera: *Candida albicans* and *Candida glabrata*. *C. albicans* is associated with roughly 50% of candidiasis with *C. glabrata* making constituting 25% of the remaining infections (Pfaller, Andes et al. 2014). *C. glabrata* has risen dramatically in frequency since the introduction of azole drugs in the 1980s, likely in part due to its facile acquisition of resistance to this antifungal drug (Wiederhold 2017) although recent findings suggest additional, less appreciated complexities in the *C. glabrata* lifestyle may also impact its development of drug resistance (Bojsen, Regenberget al. 2017, Gabaldon and Fairhead 2018). The nearly exclusive mechanism driving azole resistance in *C. glabrata* are substitution mutations within the gene encoding a key transcriptional regulator of drug resistance. This transcription factor is known as Pdr1 based on its striking sequence similarity with the homologous protein from *Saccharomyces cerevisiae* (Vermitsky and Edlind 2004). Pdr1 increases the expression of an ATP-binding cassette (ABC) transporter-encoding gene called *CDR1* in *C. glabrata* that directly confers most of the acquired azole resistance in this pathogen (reviewed in (Sanglard, Coste et al. 2009, Morschhauser 2010, Paul and Moye-Rowley 2014)).

***Saccharomyces cerevisiae* Pdr1 background**

Extensive work in *Saccharomyces cerevisiae* has provided important background for understanding of Pdr1 in *C. glabrata*. *S. cerevisiae* Pdr1 (ScPdr1) was initially identified as a locus that produced a multiple or pleiotropic drug resistant phenotype in a semidominant manner (Rank and Bech-Hansen 1973). Cloning and characterization of this gene determined that ScPdr1 was a Cys₆Zn₂ DNA-binding domain transcription factor that served as a positive regulator of genes involved in drug resistance (Balzi, Chen et al. 1987). Central among these genes is the ATP-binding cassette (ABC) transporter-encoding locus designated *PDR5* in *S. cerevisiae* (Sc*PDR5*) (Balzi, Wang et al. 1994, Bissinger and Kuchler 1994, Hirata, Yano et al. 1994). ScPdr1-dependent activation of Sc*PDR5* leads to strongly elevated drug resistance to hundreds of different compounds including azole drugs.

A key discovery to emerge from study of ScPdr1 was the nature of the semi-dominant alleles of the Sc*PDR1* gene. These were invariably substitution mutations that clustered in the carboxy-terminal region of the protein (Figure 1) (Carvajal, van den Hazel et al. 1997). Sc*PDR1* is transcribed at a constitutive level arguing that these mutant alleles enhance the ability of the protein to activate target gene transcription. A second gene encoding a homologue to ScPdr1 was found and designated Sc*PDR3* (Delaveau, Delahodde et al. 1994). This locus could also be altered to produce a hyperactive transcription factor in a fashion directly analogous to Sc*PDR1* (Figure 1) (Kean, Grant et al. 1997, Nourani, Papajova et al. 1997, Simonies, Kozovska et al. 2000). A striking difference between these two genes was the finding that transcription of Sc*PDR3* was autoregulated while that of Sc*PDR1* was not (Delahodde, Delaveau et al. 1995). Additionally, a signal from mitochondria that have lost their organellar DNA (ρ^0 cells) specifically induces the function of ScPdr3 while leaving ScPdr1 unaffected (Hallstrom and Moye-Rowley 2000, Traven, Wong et al. 2001). Genetic and biochemical analyses discovered protein factors that either negatively or positively influence ScPdr3 during ρ^0 signaling. ScPdr3 is repressed by its interaction with the Hsp70 proteins Ssa1/2 while this factor is activated through the action of the mitochondrial enzyme Psd1 (Gulshan, Schmidt et al. 2008) and the nuclear factor Lge1 (Zhang, Kolaczowska et al. 2005) (see below for details).

Regulation of *C. glabrata* Pdr1

Pdr1 was cloned from *C. glabrata* based on its sequence similarity with the same protein from *S. cerevisiae* (Vermitsky and Edlind 2004). A transposon mutagenesis screen also identified *PDR1* on the basis of azole hypersensitivity caused by an insertion into this gene (Tsai, Krol et al. 2006). Both of these studies identified alleles of *PDR1* that appeared to cause hyperactivity of the factor as seen with similar mutants in Sc*PDR1*.

These findings were confirmed in two extensive studies that explored the range of substitution mutations that were associated with increased azole resistance via presumptive changes in Pdr1 activity (Ferrari, Ischer et al. 2009, Tsai, Sammons et al. 2010). These experiments argued that many different changes in the Pdr1 amino acid sequence would lead to increased function of this transcriptional regulator.

Along with these *PDR1*-linked changes that influence activity of the factor, several other inputs act to modulate the function of Pdr1. Studies on high frequency azole resistant isolates led to the finding that, as in *S. cerevisiae*, ρ^0 cells of *C. glabrata* activated expression of ABC transporter-encoding genes leading to elevated azole resistance (Sanglard, Ischer et al. 2001). Later work established that the receptor for this signal was Pdr1 in *C. glabrata* (Vermitsky, Earhart et al. 2006). Along with the fact that there is no homologue of *PDR1* in the *C. glabrata* genome, this was a strong indication that the functions split into two loci in *S. cerevisiae*, would be combined in one gene in this pathogen. Later studies demonstrated that overproduction of the mitochondrially- localized phosphatidylserine decarboxylase Psd1 increased azole resistance in a Pdr1-and Cdr1-dependent manner (Paul, Schmidt et al. 2011). This observation provides a further link tying control of Pdr1 activity to mitochondrial functions as was observed earlier in *S. cerevisiae* for ScPdr3 (reviewed in (Moye-Rowley 2005)). Another factor that is required for normal ρ^0 -induction of ScPdr3 is the ubiquitin ligase subunit Lge1 (Hwang, Venkatasubrahmanyam et al. 2003). Experiments in *S. cerevisiae* determined that while Lge1 does participate in histone H2B ubiquitination, this function of Lge1 is not required for control of ScPdr3 in ρ^0 cells (Zhang, Kolaczowska et al. 2005). *C. glabrata* does contain a Lge1 homologue but its role in control of Pdr1 is currently unexplored. While the ρ^0 induction of Pdr1 function is a well-established phenomenon corroborated by several groups, the molecular basis of this regulation remains unknown. This is also true in *S. cerevisiae*.

Another regulator of Pdr1 activity is the azole drugs themselves. Early experiments demonstrated that the addition of itraconazole or fluconazole led to Pdr1-dependent activation of *CDR1* transcription (Vermitsky and Edlind 2004). Later work argued that Pdr1 bound directly to fluconazole and it was this binding that triggered the activation of Pdr1 function (Thakur, Arthanari et al. 2008). This was an important suggestion that would provide a basis for control of Pdr1-regulated transcription. Simple interpretation of this finding is somewhat clouded by the fact that overproduction of wild-typePdr1, in the absence of any inducer, still leads to elevated target gene expression (Tsai, Krol et al. 2006, Khakhina, Simonovicova et al. 2018). One possible explanation is that high level production of Pdr1 overcomes the normal negative regulation of this factor and leads to increased downstream gene expression. Alternatively, it is conceivable that Pdr1 accumulates in some slightly misfolded form that is capable of inducer independent gene activation. Further studies are required to resolve these possibilities.

Although at an earlier stage compared to *S. cerevisiae*, there are clear indications that trans-acting factors modulate Pdr1 activity. The first of these factors was the transcriptional Mediator component Med15A (Thakur, Arthanari et al. 2008). Mediator is a multiprotein complex that acts to link transcription factors with the RNA polymerase II machinery (recently reviewed in (Soutourina 2018)). Loss of Med15A strongly depressed Pdr1-dependent gene activation and blocked azole induction of Pdr1. Interestingly, loss of Med15A did not prevent ρ^0 -induced activation of Pdr1 (Paul, Schmidt et al. 2011), suggesting that different mechanisms may underlie drug or ρ^0 -induction via Pdr1.

More recently, a genetic approach has identified the DnaJ protein Jjj1 as a negative regulator of Pdr1 function (Whaley, Caudle et al. 2018). This is reminiscent of the situation in *S.*

cerevisiae in which the DnaK protein ScSsz1 was found to positively affect ScPdr1 (Hallstrom, Katzmann et al. 1998) transcription as was the DnaJ protein ScZud (Eisenman and Craig 2004). DnaK proteins are Hsp70 chaperones while DnaJ proteins regulate ATP hydrolysis of the associated DnaK along with providing substrate binding functions (recently reviewed in (Mogk, Bukau et al. 2018)). Extensive structural analyses were interpreted to suggest that direct binding of ScPdr1 by ScZuo1 (which also forms a complex with ScSsz1) is required to stimulate function of this transcription factor in *S. cerevisiae* (Ducett, Peterson et al. 2013).

The effect of Jjj1 in *C. glabrata* suggests a closer relationship with the function of another DnaK protein from *S. cerevisiae* called ScSsa1 that inhibits the activity of the Pdr1 homologue ScPdr3 (Shahi, Gulshan et al. 2007). ScSsa1 was co-purified from *S. cerevisiae* using a TAP-ScPdr3 fusion protein construct. ScSsa1 is one of 4 closely related DnaK proteins in *S. cerevisiae* and cells are not viable if they lack all 4 of these Hsp70 proteins (Craig, Ziegelhoffer et al. 1995). This restricted genetic analysis to use of overproduction constructs of either ScSsa1 or ScSsa2 which share >90% sequence identity. Either of these proteins strongly repressed ScPdr3 transcriptional activity (Shahi, Gulshan et al. 2007). The loss of Jjj1 increased Pdr1-dependent target gene expression but the mechanism of this increase is not currently understood.

Genetic definition of Pdr1 regulatory region

While isolation of a range of different hyperactive alleles of *PDR1* from clinical strains pinpointed residues required for normal regulation of this transcription factor, essentially no functional mapping of key domains had been done in *C. glabrata*. My laboratory recently described a mutant form of Pdr1 that lacked a large central region of this factor (Khakhina, Simonicova et al. 2018). This internal deletion mutant was constructed in a manner analogous to an earlier variant produced for ScPdr1 that also lacked the central domain of the factor (Hallstrom and Moye-Rowley 2000). This central domain is often referred to as the middle homology region (MHR) and serves to confer regulation on the Cys₆Zn₂ DNA-binding domain-containing protein within which it is embedded (Schjerling and Holmberg 1996). Deletion of the MHR from ScPdr1 yielded a constitutively active transcription factor driving downstream gene expression levels to a similar degree as gain-of-function (GOF) point mutations (Hallstrom and Moye-Rowley 2000). This transcriptional activation likely involves a 9 amino acid transactivation (TA) domain (Piskacek, Havelka et al. 2016) that was contained within a region shown to interact with the N-terminus of Med15A (Thakur, Arthanari et al. 2008) (Figure 1).

Removal of the MHR from *PDR1* in *C. glabrata* yielded a mutant factor that was not tolerated in *pdr1* cells (Khakhina, Simonicova et al. 2018). We could recover transformants expressing this mutant protein if we supplied wild-type Pdr1 which attenuated the activity of this deregulated mutant. We believe that heterodimers between a wild-type protein and the internal deletion mutant were less transcriptionally active than the homodimeric internal deletion mutant protein. Consistent with the overexpression of the internal deletion mutant resulting in toxicity, removal of the Pdr1 binding sites called Pdr1 Response Elements (PDREs) from the *PDR1* promoter also allowed this mutant factor to be tolerated as the sole

source of Pdr1. There are two PDREs that are required for autoregulatory induction of *PDR1* and their loss lowers the expression of the gene. We believe the cognate *ScPDR1* internal deletion is tolerated in *S. cerevisiae* due to this gene lacking autoregulation (Hallstrom and Moye-Rowley 2000). Finally, deletion of Med15A also allowed the plasmid expressing the internal deletion mutant to be stably maintained in a *pdr1A* background. As in the case of removing the PDREs from the *PDR1* promoter, loss of Med15A attenuates the transactivation capabilities of the mutant lacking the MHR. Taken together, these data support a model in which the MHR serves as a negative regulator of Pdr1 activity. The absence of the MHR appears to remove most if not all of the restraints on Pdr1 activity. This unregulated factor then exerts its toxic effect on the cell.

While a precise understanding of the molecular basis underlying Pdr1 activation is still elusive, I propose here a working model that is consistent with the data discussed above. Pdr1 can exist in two states with respect to its ability to activate gene transcription. The low activity state refers to the level of Pdr1-dependent gene expression supported by wild-type cells growing in the absence of azole drugs. Pdr1 does activate transcription of target genes under these conditions as is evidenced by the fact that in *pdr1Δ* cells, levels of target gene expression typically drop when compared to transcription in wild-type cells. This state of relatively low function is maintained by action of the DnaJ protein Jjj1 (Whaley, Caudle et al. 2018). Loss of Jjj1 induces expression of Pdr1 and *CDR1* transcription. Comparison of the induction of *CDR1* transcription caused by loss of *JJJ1* (~25-fold elevated) and the presence of hyperactive GOF alleles of *PDR1* (~60-fold elevated) (Ferrari, Ischer et al. 2009, Khakhina, Simonovicova et al. 2018) suggests that more than Jjj1 is required to restrain activity of Pdr1 and maintain this low activity state. We have identified a second negative regulator that directly binds to Pdr1 and are currently investigating the relationship of this factor to Jjj1 (Paul, McDonald and Moye-Rowley; unpublished data).

Three different triggers lead to increased Pdr1 function: Activation of Pdr1 can readily be triggered by the addition of azole drugs to cells. This represents an acute and reversible induction of Pdr1 function. Chronic activation of Pdr1 with the introduction of various substitution mutations across the carboxy-terminal region of the protein chain leads to robust and permanent high-level expression of target genes. As with *ScPdr3*, *p⁰ C. glabrata* strains exhibit strongly elevated levels of *PDR1* expression, target gene transcription and decreased azole susceptibility. These phenotypes all come with an associated growth defect caused by highly defective mitochondria (Contamine and Picard 2000).

Available evidence indicates different modes of action for these triggers. Loss of the mitochondrial genome causes the largest increase in levels of Pdr1 protein (~10-fold) (Paul, Bair et al. 2014) and exhibits a lack of dependence on Med15A for downstream gene activation (Paul, Schmidt et al. 2011). Either challenge with azole drugs or the presence of GOF forms of Pdr1 increase Pdr1 proteins levels by a more modest amount (~3-fold) and have varying effects on transcription of downstream target genes like *CDR1*. Drug-induced *CDR1* mRNA levels are in the 4–6-fold range (Vermitsky and Edlind 2004) while the presence of GOF alleles of *PDR1* can elevate *CDR1* mRNA to 30-fold above those in the presence of wild-type *PDR1* (Caudle, Barker et al. 2011). Taken together, these observations present a complex network of interactions that control the activity of Pdr1. The fact that the

primary mode of azole resistance in *C. glabrata* is mutational activation of *PDR1* underlines the importance of understanding the regulation of this factor. Our demonstration that a derivative of Pdr1 lacking the MHR is lethal suggests that modulation of the regulatory system of this protein might be used as a vulnerability in drug resistant isolates. The fact that mutants lacking the MHR are lethal yet GOF alleles are not is consistent with the notion that this central region of Pdr1 represents the major route of control of the transcriptional activity of this factor. Unlike the situation in GOF mutants, deletion of the MHR may remove most if not all of the negative control of Pdr1, yielding an unregulated factor that induces its own expression to a toxic level. Autoregulation of *PDR1* transcription is required for the toxicity of the internal deletion mutant consistent with this idea (Khakhina, Simoncova et al. 2018).

A central goal is the elaboration of the molecular basis explaining control of *C. glabrata* Pdr1. This factor is a blend of the properties of ScPdr1 and ScPdr3 and, while analyses of these proteins have been invaluable in the characterization of *C. glabrata* factor, it is crucial to study Pdr1 directly in *C. glabrata*. Development of modalities that interfere with the transcriptional activation by this regulatory protein have the potential to lower the high-level azole resistance supported by mutant derivatives in clinical isolates as was recently demonstrated (Nishikawa, Boeszoermyenyi et al. 2016). Understanding both the control of Pdr1 and how this factor impacts target genes will provide important new candidates for aiding in the treatment of candidemias associated with this problematic *Candida* species.

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Figure 1. Diagram of key regions in Pdr1.

A scale drawing is shown with important functional domains indicated as in the text. *C. glabrata* Pdr1 is shown as three boxes with the Cys6Zn2 cluster-containing DNA-binding domain (DBD) indicated, followed by the middle homology region (MHR) and the C-terminal transactivation (TA) domain. Two of the four regions (Tsai, Sammons et al. 2010) in which gain-of-function (GOF) mutations have been found are shown above the diagram to display the relevant amino acid residues. The one letter amino acid code is used throughout and the numbering schemes refer to the residue number from each of these three different transcription factors. The numbering at the top of the line corresponds to *C. glabrata* Pdr1. Conserved positions that are identical in at least two of these proteins are shaded red. The location of the 9 amino acid transactivation domain (9aa TAD) is shown by the bar under the sequences.

Figure 2. Model for control of Pdr1 activity.

A line drawing of *C. glabrata* Pdr1 is shown with key domains indicated as in Figure 1. Low activity refers to the ability of the protein to induce basal transcription of target genes. The DnaJ protein Jjj1 maintains Pdr1 in this low activity state. Loss of the mitochondrial genome (ρ^0 cells), the presence of azole drugs or acquisition of a GOF mutation leads to conversion of Pdr1 into a high activity form, with a higher capacity to activate gene expression. Conversion of Pdr1 from a form with the transactivation domain more tightly associated with the rest of the protein chain (low activity) to a more accessible form (high activity) could explain how the transactivation function is controlled but is only a suggestion at this time.