MINIREVIEW

Antifungal drug resistance mechanisms in fungal pathogens from the perspective of transcriptional gene regulation

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Abstract

Fungi are primitive eukaryotes and have adapted to a variety of niches during evolution. Some fungal species may interact with other life forms (plants, insects, mammals), but are considered as pathogens when they cause mild to severe diseases. Chemical control strategies have emerged with the development of several drugs with antifungal activity against pathogenic fungi. Antifungal agents have demonstrated their efficacy by improving patient health in medicine. However, fungi have counteracted antifungal agents in several cases by developing resistance mechanisms. These mechanisms rely on drug resistance genes including multidrug transporters and drug targets. Their regulation is crucial for the development of antifungal drug resistance and therefore transcriptional factors critical for their regulation are being characterized. Recent genome-wide studies have revealed complex regulatory circuits involving these genetic and transcriptional regulators. Here, we review the current understanding of the transcriptional regulation of drug resistance genes from several fungal pathogens including *Candida* and *Aspergillus* species.

Introduction

Fungi are primitive eukaryotes interacting with other life forms (bacteria, plants, insects, mammals) under specific relationships known as mutualism, parasitism or commensalism. When causing mild to severe diseases in their hosts, specific fungal species are categorized as pathogens. Most of the fungal pathogens in mammals are so-called opportunistic, because they will only cause disease when host immune defenses are deficient. In recent decades, opportunistic fungal infections have gained considerable importance due to an increase in the immunocompromised population comprising individuals infected with the HIV, patients undergoing immunosuppressive treatment in preparation for organ and bone marrow transplantation or cancer patients receiving cytotoxic agents (Richardson & Lass-Florl, 2008). *Candida albicans* is the most frequent species among the current fungal pathogens, followed by other *Candida* species (e.g. *Candida glabrata*, *Candida parapsilosis*, *Candida tropicalis*), non-*Candida* species (e.g. *Cryptococcus neoformans*) and molds (e.g. *Aspergillus fumigatus*, *Microsporum canis*) (Lass-Florl, 2009). These fungi are responsible for various forms of diseases, ranging from superficial infections of the mucosal surfaces or skin to systemic infections, which, in most cases, are life-threatening.

The treatment of human fungal diseases relies primarily on the availability of antifungal agents. While diverse antifungal agents representing eight different chemical classes exist for combating fungal pathogens encountered in crop protection and agriculture, antifungals used in medicine are divided into only seven major different chemical classes including polyenes, pyrimidine analogues, azoles, candins, allylamines, thiocarbamates and morpholines. The latter two classes, together with other agents (griseofulvin, ciclopirox, undecylenic acid), are of marginal importance in the treatment of fungal diseases (Thompson *et al.*, 2009). Azoles and thiocarbamates are the only overlapping classes between the agents used in the environment and medicine. Here, we will briefly summarize the mode of action and activity spectra of agents used in medicine.

Polyenes

The polyenes belong to a class of natural compounds with an amphipathic nature (one hydrophilic charged side of the

molecule and one hydrophobic, uncharged side of the molecule). The polyenes target ergosterol in the fungal membrane and create pores that allow small molecules to diffuse across the membrane, resulting in cell death (Canuto & Rodero, 2002). There are two main polyenes: amphotericin B and nystatin. Amphotericin B is the gold standard in the treatment of most fungal infections (*Candida*, *Cryptococcus* and *Aspergillus*), especially in severe invasive infections where a rapid response is needed.

Pyrimidine analogues

5-Fluorocytosine (5-FC) is the only representative of this class of antifungals. Susceptible fungi have a cytosine deaminase that converts 5-FC into 5-fluorouracil, which is then incorporated into DNA and RNA, therefore inhibiting cellular function and division (Polak & Scholer, 1975). 5-FC is usually used in combination with polyenes or other antifungal agents in the treatment of fungal infections because resistance develops at a high frequency as monotherapy. 5-FC has poor activity against most filamentous fungi and dermatophytes (Gehrt *et al.*, 1995; Sanglard, 2002). This can be attributed to their lack of a cytosine deaminase, which is also the basis for the minimal toxicity of 5-FC in mammalian cells (Edlind, 2007).

Azoles

Azoles, together with allylamines, thiocarbamates and morpholines, inhibit ergosterol biosynthesis, a pathway that is similar in many respects to cholesterol biosynthesis in mammals, but differs in subtle ways. Azoles inhibit a cytochrome P450 lanosterol demethylase, Erg11 or Cyp51, which is an essential step in sterol biosynthesis. Inhibition of lanosterol demethylase results in the replacement of ergosterol by methylated sterols in the plasma membrane (Sanglard, 2002). Azoles may also inhibit another cytochrome P450 responsible for sterol Δ^{22}-desaturation (Erg5), a later step in ergosterol biosynthesis (Skaggs *et al.*, 1996). Because Erg11 precedes Erg5 in sterol biosynthesis, the former enzyme is most important as an azole target. There are two classes of azole drugs: (1) the imidazoles, including ketoconazole, miconazole and clotrimazole, which have limited use for systemic infections, but are commonly used topically for mucosal or skin infections, and (2) the triazoles, including fluconazole, voriconazole, itraconazole and posaconazole, which are used systemically for both mucosal and systemic infections. Voriconazole, itraconazole and posaconazole have good activity against most filamentous fungi, in contrast to fluconazole, whose activity is largely limited to yeast (Sheehan *et al.*, 1999).

Candins

Candins belong to the most recent category of antifungal drugs. They inhibit β-1,3 glucan synthase, an enzyme complex that is located in the plasma membrane of fungal cells. This enzyme is essential to fungi as β-1,3 glucans represent one of the major components of the fungal cell wall. Several isoforms of β-1,3 glucan synthase are present in *Candida* species; however, the major activity is attributed to a single isoform (referred to as Fks1). Candins are now available as three different, but chemically related compounds: caspofungin, micafungin and anidulafungin. Candins are used for the treatment of invasive *Candida* and *Aspergillus* infections, but are not effective for *Cryptococcus* and most mold infections (Perlin, 2007).

Allylamines, thiocarbamates and morpholines

These compounds inhibit the biosynthesis of ergosterol at different steps. The allylamines (terbinafine) and thiocarbamates (tolnaftate) inhibit the same enzyme, squalene epoxidase (Erg1), which represents an early step in ergosterol biosynthesis. The morpholines (fenpropimorph) inhibit two different enzymes, Erg2 and Erg24, catalyzing sterol Δ^{14}-reductase and Δ^{8}–Δ^{7} isomerase, respectively. Although allylamines, thiocarbamates and morpholines have wide activity spectra against fungal species, they are usually used as topical agents to treat dermatophyte infections (Niewerth & Korting, 2000).

The use of antifungal agents, especially following repeated or long-term therapy, leads to the inevitable development of resistance. To determine the occurrence of resistance to these compounds, the quantification of antifungal susceptibility has been standardized using different protocols. These protocols, in which fungal growth is recorded in the presence of serial drug dilutions over a defined time period, provide a minimum inhibitory concentration (MIC) that is defined as the lowest drug concentration resulting in a significant reduction of growth (usually either 50% or 90% reduction compared with growth in the absence of the drug). The MIC breakpoint values that are used to distinguish resistant fungal isolates from susceptible isolates depend on several factors including *in vitro* laboratory or clinical observations (Arikan, 2007). Antifungal resistance is reflected *in vitro* by an increase in MIC as compared with MICs measured in control cells that are still susceptible to drugs. Because it is measured out of the clinical context, it is referred to as microbiological resistance. Clinical resistance, however, is understood as a failure of an infected patient to respond to antifungal treatment even if the maximal dosage is used. Clinical resistance to a fungal infection can be observed even when no microbiological resistance to an antifungal agent can be measured *in vitro*. Interpretative MIC breakpoints for several antifungal agents (fluconazole,

itraconazole, 5-FC, candins) have been proposed to bridge microbiological resistance with clinical resistance. However, the predictive value of these clinical breakpoints is still debated (Espinel-Ingroff, 1997; Rex *et al.*, 1997; Pfaller *et al.*, 2008).

In general, the incidence of antifungal resistance in normally susceptible fungal species is moderate, especially when compared with the incidence of antibiotic resistance among bacterial pathogens. However, antifungal resistance is a serious concern due to the limited number of available agents. It is therefore important to understand the mechanisms of resistance to antifungal agents. This may help in the design of guidelines for choosing alternative therapies. Moreover, the molecular understanding of resistance mechanisms identifies fungal genes, which can then be used for resistance detection by molecular diagnostic tools. These genes and their associated products can undergo specific modifications in the development of resistance. Besides the occurrence of mutations in drug resistance genes as a cause of antifungal resistance, transcriptional regulation of drug resistance genes is of special interest because this mechanism can be modified transiently or permanently in fungal cells. For this reason, in this review, we will summarize the current understanding of molecular resistance mechanisms to antifungal agents, but focusing more specifically on the transcriptional regulation of drug resistance genes.

Resistance mechanisms to antifungal agents

Resistance mechanisms that prevail among fungal pathogens fall into different categories: (1) transport alterations, (2) target alterations, (3) utilization of compensatory pathways and (4) presence of complex multicellular structures.

Transport alterations

Transport alterations leading to antifungal resistance are mediated through several types of transporters in fungi, for example ATP-binding cassette (ABC) transporters and major facilitators. ABC transporters are generally made up of two transmembrane domains (TMDs) and two cytoplasmic nucleotide-binding domains (NBDs). Typically, the TMDs are composed of 12 transmembrane α-helices segments (TMS) (Gaur *et al.*, 2005; Prasad *et al.*, 2006). Transport of ABC transporter substrates across the membrane requires energy from the hydrolysis of ATP carried out at the NBDs.

Candida albicans possesses two highly homologous ABC transporters, *Candida* drug resistance 1 (Cdr1) and Cdr2, which are composed of two homologous halves, each made up of a hydrophilic, cytoplasmic NBD and TMD composed of six TMS, a so-called $(NBD–TMD_6)_2$ topology. Cdr1 and Cdr2 overexpression is responsible for azole resistance in many clinical isolates recovered from patients receiving long-term antifungal therapy (Sanglard *et al.*, 1995, 1996, 1997; White, 1997; White *et al.*, 2002). The *CDR1* gene is a functional homologue of the *Saccharomyces cerevisiae* pleiotropic drug resistance gene *PDR5* and was originally cloned through its ability to complement an *S. cerevisiae* cycloheximide hypersusceptible *pdr5*Δ mutant (Prasad *et al.*, 1995). The expression of *CDR1* in this *pdr5*Δ mutant also increased resistance to many other drugs, suggesting that *CDR1* was also a multidrug resistance (*MDR*) gene. Cdr2 was also cloned by functional complementation in *S. cerevisiae* (Sanglard *et al.*, 1997). Cdr1 and Cdr2 substrates vary considerably as they include structurally unrelated compounds such as azoles, lipids and steroids (Shukla *et al.*, 2003, 2006). Deletion of both *CDR1* alleles in *C. albicans* results in high intracellular fluconazole levels. The *cdr1*Δ/Δ mutant strain is hypersensitive to azoles, terbinafine, amorolfine and several other metabolic inhibitors (cycloheximide, brefeldin A and fluphenazine). Combining both *CDR1* and *CDR2* disruption enhances drug susceptibility as compared with single mutants (Sanglard *et al.*, 1996). Experiments undertaken by several laboratories concluded that *CDR1* was a prime contributor of azole resistance in clinical isolates as compared with *CDR2* (Holmes *et al.*, 2008; Tsao *et al.*, 2009). Additional ABC transporters (e.g. Cdr3 and Cdr4) among the remaining 26 *C. albicans* ABC proteins (Gaur *et al.*, 2005) do not seem to contribute to azole resistance as shown by several studies (Balan *et al.*, 1997; Franz *et al.*, 1998).

In other *Candida* species, functional homologues of *CDR1* and *CDR2* have been described and associated with drug resistance. In *C. glabrata*, the constitutive high-level expression of the ABC-transporter genes *CgCDR1*, *CgCDR2* (also known as *PDH1*) and *CgSNQ2* plays a dominant role in azole resistance (Miyazaki *et al.*, 1998; Sanglard *et al.*, 1999, 2001; Bennett *et al.*, 2004; Vermitsky & Edlind, 2004; Torelli *et al.*, 2008). One or more of these genes are commonly upregulated in azole-resistant clinical isolates. The upregulation of *CgCDR1*, *CgCDR2* and *CgSNQ2* is associated with mutations in a transcriptional regulator (see below); however, it may also be due to mitochondrial deficiencies. *Candida glabrata* is prone to spontaneous or induced mitochondrial modifications. They result in strong transcriptional changes of ABC transporter genes and therefore mediate antifungal resistance (Sanglard *et al.*, 2001; Brun *et al.*, 2003, 2004).

Other ABC transporters from *Candida dubliniensis* (*CdCDR1* and *CdCDR2*), *Candida krusei* (*ABC1* and *2*), *C. tropicalis* (*CDR1* homologue) and from *C. neoformans* (*CnAFR1*, antifungal resistance 1) were reported to be upregulated in azole-resistant isolates (Moran *et al.*, 1998; Barchiesi *et al.*, 2000; Katiyar & Edlind, 2001; Posteraro *et al.*, 2003; Pinjon *et al.*, 2005). *ABC1* from *C. krusei* was expressed in *S. cerevisiae* and shown to confer resistance to

azoles. However, because Erg11 from *C. krusei* is less sensitive to azoles than other fungal orthologues, *ABC1* is not the only mediator of azole resistance in this yeast species (Lamping *et al.*, 2009). In *A. fumigatus*, *atrF* and *AfuMDR4* are upregulated in itraconazole-resistant strains (Nascimento *et al.*, 2003). To date, only *CdCDR1* and *CnAFR1* have been experimentally associated with azole resistance (Moran *et al.*, 1998; Posteraro *et al.*, 2003).

The second class of transporters involved in antifungal resistance belongs to the major facilitator superfamily (MFS). MFS transporters are ubiquitously present in all kingdoms of life. They are involved in the symport, antiport or uniport of various substrates. Most MFS proteins vary between 400 and 600 amino acid residues in length and possess either 12 or 14 putative TMS with an intercalating cytoplasmic loop. The MFS consists of 61 families. In yeast, the MFS transporters involved in drug resistance function by proton antiport and are classified into two groups: the drug: H+ antiporter-1 (12 TMS) (DHA1) family and the drug: H+ antiporter-2 (14 TMS) (DHA2) family (Gaur *et al.*, 2008; Sa-Correia *et al.*, 2009).

The MFS transporter gene *MDR1* (formerly *BEN*^{r} for benomyl resistance) of *C. albicans* is a member of the DHA1 family and was originally cloned for its ability to confer resistance to several compounds including benomyl, methotrexate, cycloheximide, benztriazole and 4-NQO when expressed in *S. cerevisiae* (Fling *et al.*, 1991; Ben-Yaacov *et al.*, 1994). Clinical *C. albicans* isolates overexpressing *MDR1* are more resistant to drugs such as fluconazole, 4-NQO, cerulenin and brefeldin A, as compared with matched isolates with no *MDR1* detectable expression (Wirsching *et al.*, 2001). In contrast to the overexpression of *CDR* genes rendering cells resistant to many different azoles, overexpression of *MDR1* appears to be specific to fluconazole and is not associated with cross-resistance to other azoles (Sanglard *et al.*, 1995, 1996, 1997). Homologues of *MDR1* in *C. dubliniensis* and *C. tropicalis*, named *CdMDR1* and *CtMDR1*, respectively, are upregulated in azole-resistant strains (Barchiesi *et al.*, 2000; Wirsching *et al.*, 2001). Because the *CdCDR1* gene is inactivated by a point mutation in many *C. dubliniensis* strains and *CdCDR2* is poorly expressed, *CdMDR1* overexpression is the major remaining mechanism of fluconazole resistance in this species (Moran *et al.*, 1998, 2002; Wirsching *et al.*, 2001). *CdMDR1* inactivation does not cause increased susceptibility to amorolfine, terbinafine, fluphenazine or benomyl (Wirsching *et al.*, 2001).

In *C. glabrata*, the *MDR1* homologue is *CgFLR1*, which is closely related to *FLR1* in *S. cerevisiae* (Chen *et al.*, 2007). *FLR1* is under the control of the transcription factor *YAP1* and is able to confer fluconazole resistance when *YAP1* is activated (Alarco *et al.*, 1997). Although *CgFLR1* can mediate fluconazole resistance when expressed in *S. cerevisiae*, the significance of *CgFLR1* in azole resistance in *C. glabrata* has not yet been demonstrated as it is probably masked by the vast majority of azole-resistant isolates upregulating ABC transporters (Sanglard *et al.*, 1999). In *A. fumigatus*, *in vitro*-generated itraconazole-resistant isolates show a constitutive high-level expression of the MFS transporter *AfuMDR3* (Nascimento *et al.*, 2003).

A *C. albicans* gene encoding a second MFS transporter, *FLU1* (fluconazole resistance), was isolated by complementation of the azole hypersusceptible *S. cerevisiae pdr5*Δ mutant (Calabrese *et al.*, 2000). The expression of *FLU1* in this strain mediated not only resistance to fluconazole but also to cycloheximide among the different drugs tested. The disruption of *FLU1* in *C. albicans* had only a slight effect on fluconazole susceptibility, but disruption of *FLU1* in a mutant with deletions in several multidrug efflux transporter genes, including *CDR1*, *CDR2* and *MDR1*, resulted in enhanced susceptibility to several azole derivatives, demonstrating that *FLU1* can mediate azole resistance in *C. albicans*. However, *FLU1* overexpression has not yet been identified as a cause of azole resistance in clinical isolates (Calabrese *et al.*, 2000).

Target alterations by mutations and gene upregulation

Target alterations are known resistance mechanisms for two classes of antifungal agents: azoles and candins. Resistance mediated by alterations in Erg11/Cyp51 (targets of azoles) has been widely documented involving either mutations or upregulation of their genes. A large number of nonsynonymous nucleotide polymorphisms (up to 110, including 100 with unique substitutions) have been described in *ERG11* alleles originating from *C. albicans* azole-resistant isolates. The degree of *ERG11* polymorphism is therefore high and suggests that Erg11 is highly permissive to structural changes resulting from amino acid substitutions. The contribution of each individual mutation to azole resistance is, however, difficult to estimate because *ERG11* mutations often occur in combination (from two to four combined mutations) in the same allele and because resistance mechanisms are often combined in azole-resistant *C. albicans* isolates (Marichal *et al.*, 1999). Using different approaches (heterologous expression in *S. cerevisiae*, enzyme assay in *C. albicans* extracts, site directed mutagenesis), evidence for their involvement in azole resistance has been provided for at least some of these mutations (F72L, F145L, G464S, Y132F, R467K, S405F) (Sanglard *et al.*, 1998; Asai *et al.*, 1999; Favre *et al.*, 1999; Kelly *et al.*, 1999a, b; Lamb *et al.*, 2000; Kudo *et al.*, 2005).

ERG11 upregulation has often been associated with azole resistance in *C. albicans* (Sanglard *et al.*, 1995; Marichal *et al.*, 1997; White, 1997; Perea *et al.*, 2001) or in a single

C. tropicalis isolate (Vandeputte *et al.*, 2005). This resistance mechanism involves a gene dosage effect, in which the increased Erg11 numbers of molecules preclude saturation with azoles.

In *A. fumigatus*, itraconazole resistance in clinical isolates is associated with the occurrence of amino acid substitution in Cyp51A, which is the functional orthologue of Erg11 in this fungal species. Interestingly, mutations at position G54 contribute only to itraconazole resistance and not to voriconazole resistance (Diaz-Guerra *et al.*, 2003; Mann *et al.*, 2003). In contrast, mutations at position M220 confer itraconazole resistance and also high MICs to voriconazole or posaconazole (Garcia-Effron *et al.*, 2008a). Similarly, mutations at positions L98 and G138 recently described in Cyp51A conferred cross-resistance to all azoles (Garcia-Effron *et al.*, 2008a, b, c). Interestingly, the Cyp51A mutation L98H is consistently combined with *cyp51*A upregulation. This mechanism allows cross-resistance to all known azoles (Mellado *et al.*, 2007). Intriguingly, the L98H substitution and *cyp51*A upregulation mechanisms were also found in isolates of environmental origin, thus raising the question of how azole resistance was acquired in a nonmedical environment (Snelders *et al.*, 2008).

In *C. neoformans*, analysis of *ERG11* from a clinical azole-resistant isolate showed that a point mutation linked an amino acid substitution G484S that was not observed in the parent azole-susceptible isolates (Rodero *et al.*, 2003). Recent studies demonstrated that azole resistance in this yeast species can be due to hetero-resistance, which is a mechanism by which resistance can be induced or reversed in a portion of a growing population (Sionov *et al.*, 2009). Hetero-resistance in *C. neoformans* appears to involve chromosomal aneuploidies, and therefore gene copy number increase of drug resistance genes including *ERG11*. This mechanism can therefore result in elevated *ERG11* transcription (J. Kwon-Chung, pers. commun.).

The absence of a target can also lead to antifungal resistance. For example, nonfunctional Erg6 in *C. glabrata* was reported to be associated with amphotericin B resistance. Because no ergosterol is present in the plasma membranes of Erg6^{-}-defective mutants, amphotericin B cannot exert its inhibition, which otherwise requires ergosterol for pore formation in the plasma membrane (Vandeputte *et al.*, 2007, 2008).

The introduction of the class of candins in the treatment of fungal diseases was followed shortly by the isolation of resistant clinical isolates, although at very low frequencies. Target alterations have been observed conferring candin resistance at the level of the enzyme β-1,3 glucan synthase (*FKS1*), and similar mutations were obtained by *in vitro* selection after exposure to the drug (Douglas *et al.*, 1997; Park *et al.*, 2005). These mutations are located in two hotspot regions (HS1, HS2); however, HS1 (located between residues 641 and 649 of the *C. albicans* Fks1) is the region with the most substitutions (Perlin, 2007). These mutations generally cause cross-resistance to all three known candins. *FKS1* modifications in HS1 domains have also been detected in other species including *C. tropicalis* (Garcia-Effron *et al.*, 2008b, c), *C. glabrata* (Cleary *et al.*, 2008) and *A. fumigatus* (Rocha *et al.*, 2007). The intrinsic reduced susceptibility of *C. parapsilosis* is also attributed to natural substitution in the HS1 domain (Garcia-Effron *et al.*, 2008b, c). Several β-1,3 glucan synthase subunits exist in fungal genomes and therefore mutations in these additional genes can also be targeted by mutations. For example, substitutions in Fks2 from *C. glabrata* are associated with caspofungin resistance (Katiyar *et al.*, 2006; Thompson *et al.*, 2008).

Little is known about the relationship between the altered expression of candin target genes and resistance. It was reported that the activation of the cell integrity pathway by exposure to caspofungin can result in enhanced expression of *FKS* genes (Reinoso-Martin *et al.*, 2003). No intrinsic overexpression of *FKS* genes in fungal pathogens has been associated as yet with the acquisition of candin resistance.

Utilization of compensatory and catabolic pathways

In some instances, yeast cells can modify to their advantage the biosynthesis of otherwise toxic metabolites that are derived from exposure to antifungal agents. The development of azole resistance by mutations in *ERG3* encoding sterol $\Delta^{5,6}$ desaturase is an illustration of this principle. If active, Erg3 converts 14α-methylated sterols into toxic 3,6-diol derivatives (Kelly *et al.*, 1995). Fungal cells unable to produce this toxic metabolite acquire azole resistance. Consistent with this observation, fungal azole-resistant isolates with nonfunctional *ERG3* alleles have been described in *C. albicans* and *C. dubliniensis* (Pinjon *et al.*, 2003; Chau *et al.*, 2005). Inactivation of *ERG3* (as well as other *ERG* genes) is usually associated with differential regulation of genes involved in the ergosterol biosynthetic pathway. This is probably the consequence of absence of ergosterol feedback inhibition on the genes of this pathway (Sanglard *et al.*, 2003).

Diversion of toxic metabolite formation is also observed in the development of resistance to 5-FC, a compound that is normally metabolized by cells into fluorinated pyrimidine analogues interfering with nucleic acid and protein biosynthesis. A mutation in the *FUR1* gene encoding uracil phosphoribosyltransferase is thought to decrease the conversion of 5-fluorouridine, which is produced from deamination of 5-FC, into a toxic metabolite (5-fluorouridine monophosphate), and thus counteracts the action of this compound (Dodgson *et al.*, 2004). Mechanisms other than *FUR1* inactivation can be operative in *C. albicans* as

suggested by early studies performed in *C. albicans* and *C. glabrata*. Deficiencies in cytosine permease, cytosine deaminase or alterations in thymidylate synthase activities are all possible (Vanden Bossche *et al.*, 1994). Consistent with this hypothesis, clinical isolates showing 5-FC resistance that are homozygous for a mutation in cytosine deaminase (*FCA1*) have been reported (Hope *et al.*, 2004).

Antifungal resistance by the presence of complex multicellular structures (biofilms)

Biofilms are formed on synthetic or natural surfaces by a dense network of yeast and filaments generally embedded in an extracellular matrix (Chandra *et al.*, 2001; Ramage *et al.*, 2002). Biofilms can form on synthetic materials such as those present in catheters and are therefore of significant clinical relevance. Biofilms are formed by several fungal species including *Candida* and *Aspergillus* species. One remarkable feature of biofilms is their resistance to many unrelated antifungal agents, with the exception of caspofungin (Ramage *et al.*, 2002). The molecular basis for antifungal resistance in biofilms is still poorly understood, although several explanations have been provided. Recent published studies suggest that biofilms contain variable proportions of persister cells (phenotypic variants) that are more tolerant to drug action (LaFleur *et al.*, 2006). The term tolerance is used here to indicate that these cells have the ability to survive drug action without expressing or using resistance mechanisms, as defined by Lewis (2007). Biofilms also contain a heterogeneous cell population at different growth stages with each different transcriptional activity of genes known to be involved in drug resistance (e.g. *ERG11*, *CDR1*, *CDR2*, *MDR1*), and can thus contribute transiently to drug resistance (Mukherjee *et al.*, 2003; Cao *et al.*, 2005; Borecka-Melkusova *et al.*, 2009). Biofilms can also sequester antifungal agents (azoles, amphotericin B) in the polymers of the matrix and thus neutralize their inhibitory effects (Nett *et al.*, 2007).

Regulation of drug resistance genes in fungal pathogens

Transcriptional regulation is of pivotal importance in the development of antifungal resistance. Given that the acquisition of antifungal resistance from a susceptible fungal species is mediated in most cases by drug exposure, the resulting transient gene expression changes are important steps that shape fungal response. Drug removal will in general reverse transcription to normal levels. Antifungal pressure, especially when exerted in the long term, will eventually lead to the appearance of mutations or chromosomal rearrangements in fungal cells. These events can affect drug resistance genes, whose expression in turn will determine the degree of acquired antifungal resistance. It is therefore important to understand the regulatory network controlling drug resistance in fungal pathogens. Below, we will further discuss the current knowledge on known *cis*- and *trans*-acting factors of drug resistance genes in fungal pathogens and how regulatory networks integrate these factors.

Regulation of multidrug transporters

The isolation of regulators of multidrug transporters in *C. albicans* has been based on different strategies. One emerged through the analysis of *cis*-acting elements in *CDR1*/*CDR2* and *MDR1*, another by sequence comparisons with functionally similar regulators, while others were deduced from genome-wide transcriptional analysis of *MDR1*-upregulating strains.

ABC transporters

The systematic dissection of the *CDR1* and *CDR2* promoters allowed the identification of five distinct regulatory elements (Fig. 1): the basal expression element (BEE) responsible for basal expression, the drug-responsive element (DRE) required for the response to drugs such as

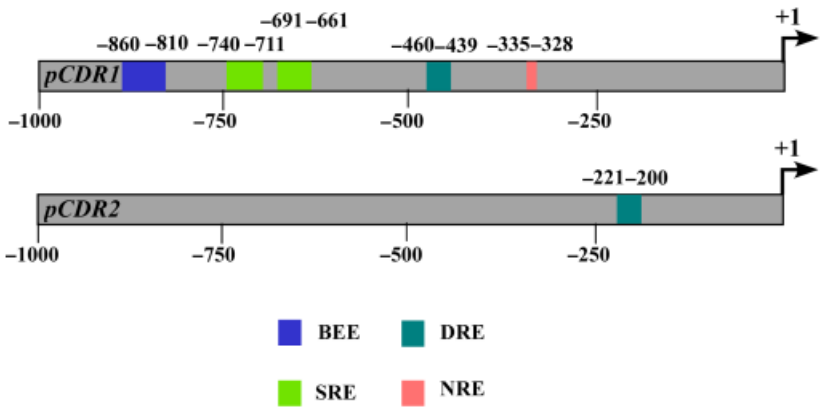

Fig. 1. Regulatory regions of the *CDR1* and *CDR2* promoters. The DRE and BEE were delimited by De Micheli *et al.* (2002). The SREs and NRE were identified by Karnani *et al.* (2004) and Gaur *et al.* (2004).

fluphenazine and estradiol, two steroid-responsive element (SRE) involved in the response to steroid hormones and the negative regulatory element (NRE) (De Micheli *et al*., 2002; Gaur *et al*., 2004; Karnani *et al*., 2004). Internal deletions of the BEE and DRE in the *CDR1* promoter affect basal *CDR1* expression and drug-induced expression, respectively. Conversely, the deletion of the NRE leads to an increased basal expression of *CDR1*. In contrast to *CDR1*, the *CDR2* promoter only contains the DRE element (De Micheli *et al*., 2002). Among these different *cis*-acting elements, only the DRE was shown to be involved not only in the transient upregulation of both *CDR1* and *CDR2* in response to inducers but also in their constitutive high expression in azole-resistant clinical isolates (De Micheli *et al*., 2002). The DREs present in the promoter of *CDR* genes contain two CGG triplets that are potentially recognized by Zn_2-Cys_6 transcription factors (Schjerling & Holmberg, 1996; Mendizabal *et al*., 1998; Hikkel *et al*., 2003; Kren *et al*., 2003). The *C. albicans* genome was searched for genes encoding proteins with Zn_2-Cys_6 finger motifs as potential *CDR1*/*CDR2* regulators. Interestingly, three of these genes were arranged in tandem near the mating-type locus, whose homozygosity is linked to the development of azole resistance in *C. albicans* (Rustad *et al*., 2002). Deletion in an azole-susceptible strain of one of these genes, transcriptional activator of *CDR* (*TAC1*) genes, led to increased drug susceptibility and to loss of transient *CDR1*/*CDR2* upregulation in the presence of inducers. In *C. albicans* clinical isolates resistant to azoles, deletion of *TAC1* abolished *CDR1*/*CDR2* expression and therefore drug resistance, thus demonstrating that *TAC1* was a major mediator of azole resistance due to the upregulation of the ABC transporter in Coste *et al*. (2004). Tac1 acts by direct binding to the DRE present in the promoter region of both efflux pump genes and induces their expression in response to steroid and several toxic chemicals (De Micheli *et al*., 2002; Coste *et al*., 2004). Tac1 is, however, not involved in the basal expression of *CDR1* and the transcription factor regulating *CDR1* expression through the BEE element remains to be identified.

Other potential regulators of *CDR1* have been reported. They were identified through functional complementation in *S. cerevisiae*. Fluconazole resistance 1 (*FCR1*) was reported as a *C. albicans* functional homologue of *PDR1*/*PDR3* in *S. cerevisiae*. *FCR1*, which belongs to the Zn_2-Cys_6 transcription factor family, was able to restore the expression of the ABC transporter *PDR5*, which is regulated by both *PDR1* and *PDR3* in *S. cerevisiae*. The deletion of *FCR1* in *C. albicans* resulted in decreased susceptibility to fluconazole (Talibi & Raymond, 1999). It is not yet clear whether *FCR1* directly regulates *CDR1* in *C. albicans*; however, a recent study revealed that *FCR1* inhibited *CDR1* induction in the presence of fluconazole (Shen *et al*., 2007). *FCR1* may therefore be a transcriptional repressor of *CDR1*. Intriguingly, the *FCR1* behavior is reminiscent to observations with the *PDR1*/*PDR3* regulators of the same family in *S. cerevisiae*. In this yeast species, the presence of *PDR3* reduces the *PDR1*-mediated *PDR5* induction in a manner similar to the *FCR1* effect on *CDR1* (Kolaczkowska *et al*., 2008). *NDT80* was another *C. albicans* gene similar to the *S. cerevisiae NDT80* gene, a meiosis-specific gene belonging to the immunoglobulin-fold family of transcription factors (Lamoureux *et al*., 2002). It was identified by its ability to regulate *CDR1* in *S. cerevisiae*. *NDT80* inactivation in *C. albicans* did result in a decreased basal *CDR1* expression and a decreased *CDR1* inducibility in the presence of drugs (Chen *et al*., 2004). *NDT80* may therefore be a positive regulator of *CDR1*. From these studies, it is not yet clear whether *NDT80* can bind to the *CDR1* promoter; however, recent studies based on novel technologies helped to answer this question.

Besides *C. albicans* ABC-transporter regulation, little is known in other fungal pathogens, with the exception of *C. glabrata*. Because of its closer relationship with *S. cerevisiae* than other fungal pathogens, ABC-transporter regulation, with regard to the identification of *cis*-acting and *trans*-acting factors, was essentially investigated by similar approaches. For example, pleiotropic drug response elements (PDREs), which control the regulation of *PDR5* in *S. cerevisiae* and harbor the consensus TCC(G/A)(C/T)G(GC)(AG), were identified in the three major ABC transporters that are responsible for azole resistance in *C. glabrata* (Sanglard *et al*., 1999; Torelli *et al*., 2008). Second, *CgPDR1* was identified from the *C. glabrata* genome as the closest homologue of *PDR1*/*PDR3*, and a fluconazole-resistant laboratory mutant allele (P927L) was isolated (Vermitsky & Edlind, 2004). This gene was later confirmed to be a functional homologue of the *S. cerevisiae PDR1*/*PDR3* genes because its inactivation in *C. glabrata* compromised the ability of ABC transporters to be upregulated in clinical isolates (Tsai *et al*., 2006; Vermitsky *et al*., 2006; Ferrari *et al*., 2009). *Cis*-acting elements regulating *CgCDR1* are currently being investigated. *CgCDR1* possess six sequences resembling the PDRE consensus. Maximal expression of *CgPDR1* is obtained when at least three PDREs are present in the *CgCDR1* promoter. Additional elements (6-bp repeats) were identified in this promoter; however, these elements are only required for *CgCDR1* expression in the presence of a wild-type *CgPDR1* allele (Raj & Edlind, 2008).

Major facilitators

Functional dissection studies of the *MDR1* promoter have identified distinct *cis*-acting regulatory regions (Fig. 2). Four different studies converged with more or less precision to an important functional element called the benomyl response element (BRE) or the *MDR1* drug resistance element

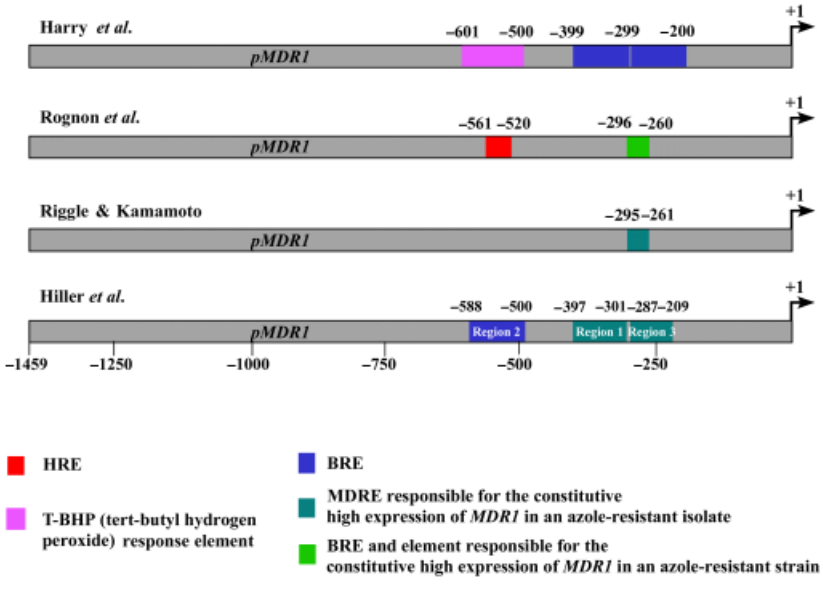

Fig. 2. Regulatory regions of the *MDR1* promoter. Promoter elements are aligned according to the study published by Harry *et al.* (2005), Rognon *et al.* (2006), Riggle & Kumamoto (2006) and Hiller *et al.* (2006). Corresponding regulatory elements and their positions are given.

(MDRE) (Harry *et al.*, 2005; Hiller *et al.*, 2006; Riggle & Kumamoto, 2006; Rognon *et al.*, 2006). This region was shown to be responsible for the constitutive high expression of *MDR1* in fluconazole-resistant isolates (Hiller *et al.*, 2006; Riggle & Kumamoto, 2006), but is also necessary for the inducible expression of *MDR1* in response to benomyl (Harry *et al.*, 2005; Rognon *et al.*, 2006). Hiller *et al.* (2006) subdivided this region into two elements (regions 1 and 3, Fig. 2). The second element, the H_2O_2 response element (HRE), is important for *MDR1* upregulation in the presence of oxidative stress agents such as tert-butyl hydrogen peroxide (Harry *et al.*, 2005). In contrast to the BRE, the HRE is not required for constitutive upregulation of *MDR1* in azole-resistant isolates. In the study of Hiller *et al.* (2006), a domain called 'region 3' contained the HRE; however, in this study, it was mediating the response to benomyl (Fig. 2). The HRE region contains two *YAP1* response element motifs and the BRE/MDRE contains a perfect match for the Mads-box transcription factor Mcm1 (Nguyen *et al.*, 2001; Harry *et al.*, 2005; Riggle & Kumamoto, 2006). While no direct evidence exists for interactions between Yap1p and the HRE, one report demonstrated recently that Mcm1 binds to the *MDR1* promoter, although at a still unknown precise location (Lavoie *et al.*, 2008). The role of Mcm1 in the inducible or the constitutive expression of *MDR1* is still unclear. A recent study has identified *NDT80* as a negative regulator of *MDR1* (Chen *et al.*, 2009). In a strain lacking *NDT80*, *MDR1* response to miconazole was increased by 10-fold as compared with the wild type. It is not yet clear whether this factor acts directly or indirectly as a transcriptional activator of *MDR1*.

The identification of an *MDR1* regulator or of factors binding to the *MDR1* promoter was, however, accomplished by genome-wide transcription profiling. By comparing the transcriptional profiles of three different *C. albicans* clinical isolates overexpressing *MDR1* with azole-susceptible parents, one of the commonly upregulated genes in the three isolates was orf19.7372. It contained a Zn_2-Cys_6 zinc finger motif. Because inactivation of orf19.7372 caused loss of *MDR1* upregulation in a clinical strain, the transcription factor was called multidrug resistance regulator 1 (Mrr1) (Morschhauser *et al.*, 2007). *MRR1* inactivation in azole-resistant isolates resulted in the loss of *MDR1* expression and increased susceptibility to fluconazole, cerulenin and brefeldin A (Morschhauser *et al.*, 2007). Deletion of *MRR1* in a drug-susceptible strain abolished *MDR1* upregulation in the presence of inducing chemicals such as benomyl and H_2O_2, thus demonstrating that Mrr1 mediates both inducible *MDR1* expression and constitutive *MDR1* upregulation in drug-resistant strains (Morschhauser *et al.*, 2007). Although Mrr1 has not been shown to bind directly to the *MDR1* promoter as yet, it is likely that this transcription factor binds directly or indirectly the regions identified as BRE or MDRE. Consistent with this hypothesis is the presence of CGG triplets in these promoter elements.

MDR1 can also be regulated by additional transcription factors. A recent study reported that regulator of efflux pump 1 (*REP1*), which belongs to the transcription factor family including *NDT80*, was acting as a negative regulator of *MDR1*. *REP1* was first isolated from a genetic screen in *S. cerevisiae*, which resulted in increased fluconazole resistance in this yeast species. When deleted in *C. albicans*, *REP1* decreased azole susceptibility and also resulted in increased expression of *MDR1* in the presence of an inducer. Interestingly, in the absence of both *REP1* and *MRR1*, *MDR1* could still be upregulated in the presence of a drug. It will be

interesting to see how Rep1 binds the *MDR1* promoter (Chen *et al*., 2009).

Regulation of antifungal target genes

The upregulation of *ERG11*, encoding a cytochrome P450 and a target of azoles, can contribute to the development of azole resistance in *C. albicans*. In *S. cerevisiae*, *ERG11* is regulated by two transcriptional activators, Upc2 and Ecm22, which are members of the Zn_2-Cys_6 transcription factor family (Vik & Rine, 2001). They act through binding to regulatory elements present in the *ERG11* promoter called sterol regulatory element (SRE). Other SREs are found in genes involved in sterol biosynthesis. A single *C. albicans* gene (*UPC2*) with homology to both *S. cerevisiae* genes has been identified and characterized (Silver *et al*., 2004; MacPherson *et al*., 2005). Deletion of *UPC2* in *C. albicans* caused loss of *ERG11* upregulation in response to azole drugs, which occurs otherwise in the parent strain. Promoter deletions and linker scan mutations localized the region important for azole induction to a segment from -224 to -251 upstream of the start codon. This segment contains two 7-bp sequences (5′-TCGTATA-3′) separated by 13 bp (Oliver *et al*., 2007), forming an imperfect inverted repeat, a typical feature for binding to Zn_2-Cys_6 transcription factors (MacPherson *et al*., 2006). The Upc2 core binding sequence is conserved between *Candida* and *Saccharomyces*. This core is found in the *ERG11* promoter in a region identified as important for azole induction of *ERG11* expression (Silver *et al*., 2004).

As mentioned above, upregulation of *cyp51A* in *A. fumigatus* has been detected in clinical isolates with cross-resistance to several azole antifungal agents. This upregulation is associated with an L98H substitution in Cyp51A and with the presence of a 34-bp tandem repeat in the *cyp51A* promoter (Mellado *et al*., 2007). This resistance mechanism has also been identified in *A. fumigatus* isolates originating from the environment in the Netherlands. Exposure of environmental isolates to agricultural azole fungicides is suspected as a possible cause of the emergence of such azole-resistant isolates recovered from treated patients (Snelders *et al*., 2008).

Transcriptional coactivators involved in drug resistance

In eukaryotes, transcription factors are part of larger complexes integrating DNA-binding proteins and the RNA polymerase machinery that are necessary for gene transcription. This is also the case for regulators of drug resistance genes. A recent elegant study has demonstrated the role of the Mediator complex in the transcriptional response of multidrug transporter genes in *S. cerevisiae* and *C. glabrata*. One subunit of this complex, Gal11, was shown to bind Pdr1 of *S. cerevisiae*. Data presented suggest that Pdr1 can act as a nuclear receptor for drugs that stimulates its association with Gal11 as a step for Mediator recruitment (Thakur *et al*., 2008). This binding is crucial for the upregulation of Pdr1 target genes. This process was conserved in *C. glabrata*. In *C. albicans*, although a *GAL11* homologue exists, the binding to the transcriptional activator of drug resistance genes (*TAC1*, *MRR1* and *UPC2*) still remains hypothetical.

The Mediator complex can associate with other transcriptional coactivators, one of which is the Spt-Ada-Gcn5 histone acetyl-transferase (SAGA) coactivator complex. The transcriptional adapter Ada2, which is part of the SAGA coactivator complex, has been shown to bind directly to both *CDR1* and *MDR1* promoters in *C. albicans* (Sellam *et al*., 2009a). Ada2 is recruited by *MRR1* to the *MDR1* promoter. In contrast, which transcription factor is recruiting Ada2 to the *CDR1* promoter is still unclear. Nevertheless, deletion of *ADA2* abolishes *CDR1* expression in response to fluconazole (Sellam *et al*., 2009a). Given these novel observations, it is likely that an association between the Mediator- and SAGA complexes could exist on promoters of *TAC1*-regulated genes. Together with transcription factors, these complexes aid the recruitment of the RNA polymerase, which itself results in the transcription of target genes (Daniel & Grant, 2007). Curiously, the histone deacetylase inhibitor trichostatin A reduces *CDR1* and *ERG11* drug-dependent upregulation in *C. albicans*, and correspondingly increases fluconazole susceptibility (Smith & Edlind, 2002). Perhaps this inhibitor is inducing the expression of a transcriptional repressor for these genes. Clearly, despite all theses recent discoveries, the molecular details that dictate the function of the transcriptional machinery regulating drug resistance genes are still at a preliminary stage, but constitute a strong basis for future studies.

Gain-of-function (GOF) mutations in transcriptional activators of drug resistance genes

The elevated transcription of genes targeted by the transcription factors *TAC1*, *MRR1*, *CgPDR1* and *UPC2* that is observed in azole-resistant clinical isolates is thought to be due to their intrinsic activation. This state of activation, which does not require external stimuli, can be obtained when the transcription factors are modified by mutations, as it is known in several other microorganisms (Carvajal *et al*., 1997; MacPherson *et al*., 2006). Consistent with this hypothesis, transcription factor alleles from azole-resistant isolates were shown to confer constitutive high expression of their drug resistance gene targets and thus azole resistance when expressed in an azole-susceptible background (Coste *et al*., 2004, 2006; Tsai *et al*., 2006; Vermitsky *et al*., 2006;

Morschhauser *et al.*, 2007; Dunkel *et al.*, 2008a, b; Torelli *et al.*, 2008; Ferrari *et al.*, 2009). This was first demonstrated in *C. albicans* in which two types of *TAC1* alleles were isolated from clinical isolates: wild-type alleles, which conferred transient *CDR1* and *CDR2* upregulation in response to drugs, and hyperactive alleles, which were isolated from azole-resistant strains and conferred constitutive high *CDR1* and *CDR2* expression and therefore drug resistance to a mutant strain lacking *TAC1* (Coste *et al.*, 2004, 2006). Sequencing of these alleles revealed that wild-type and hyperactive alleles differed by single point mutations, leading to single amino acid substitutions defined as GOF mutations. Several hyperactive alleles from *MRR1*, *CgPDR1* and *UPC2* were identified and harbored such GOF mutations. By increasing the number of investigated isolates, the number of GOF for each gene has also increased considerably. Large-scale sequencing of *TAC1* alleles from *C. albicans* clinical isolates has to date identified 39 hyperactive alleles harboring 16 different GOF mutations at 12 distinct positions. Three other GOF mutations introduced by random mutagenesis were also able to confer hyperactivity to a *TAC1* wild-type allele (Coste *et al.*, 2004, 2006; Znaidi *et al.*, 2007; Coste *et al.*, 2009) (Fig. 3). The majority of these GOF mutations (15) are located in the C-terminal portion of *TAC1* corresponding to a putative transcriptional activation domain, while the remaining mutations are situated in the middle homology region (MHR) and the N-terminal part of the protein, which are regions with no defined function (Fig. 3). How these mutations affect the transcriptional activity of *TAC1* remains unknown. Although other transcription factors have been shown to regulate *CDR1* expression in laboratory studies (Chen *et al.*, 2004; Gaur *et al.*, 2004), only mutations in Tac1 have been found to be responsible for *CDR1* and *CDR2* upregulation in clinical *C. albicans* azole-resistant isolates.

Similar to *CDR1/2* upregulation by Tac1, *MDR1* overexpression is also caused by GOF mutations in its regulator, Mrr1 (Morschhauser *et al.*, 2007). So far, 14 distinct GOF have been identified in *MRR1* on 13 distinct positions spanning throughout the ORF (Fig. 3) (Dunkel *et al.*, 2008a). As for Tac1, the functional domains of Mrr1 are still unknown. It is therefore difficult to speculate about the molecular mechanism underlying Mrr1 hyperactivity. Nevertheless, GOF mutations were also identified in *CdMRR1*, the *MRR1* homologue of *C. dubliniensis*, indicating that GOF mutations affect the activity of Mrr1 similarly in both *C. albicans* and *C. dubliniensis* (Schubert *et al.*, 2008).

Candida glabrata differs from *C. albicans* with respect to the diversity of GOF mutations in *CgPDR1* (Ferrari *et al.*, 2009). Three studies have identified four separate amino acid substitutions in CgPdr1 of azole-resistant strains that are responsible for the constitutive high expression of ABC-transporter genes and of *CgPDR1* itself (Tsai *et al.*, 2006; Vermitsky *et al.*, 2006; Torelli *et al.*, 2008). Large-scale analysis of *CgPDR1* alleles from *C. glabrata* clinical azole-resistant isolates identified 70 alleles, from which only 12 were wild-type alleles and 58 were hyperactive alleles. These 58 hyperactive alleles contain 58 distinct GOF mutations, yielding 57 single amino acids substitutions located at 51 different positions along the protein, with some 'hot spots' near the N-terminal inhibitory domain, the central MHR domain and the C-terminal activation domain (Ferrari *et al.*, 2009). The localization of CgPdr1 mutations is similar to GOF mutations described in *S. cerevisiae* homologues Pdr1/Pdr3 (Fig. 3).

To date, a single *UPC2* GOF has been described in *C. albicans* from an isolate exhibiting high *ERG11* expression levels. Using genome-wide gene expression profiling, it was revealed that *UPC2* and other genes involved in ergosterol biosynthesis were coordinately upregulated with *ERG11* in a fluconazole-resistant clinical isolate compared with a matched susceptible isolate from the same patient (Dunkel *et al.*, 2008b). Sequence analysis revealed that the resistant isolate contained a single-nucleotide substitution in one *UPC2* allele that resulted in a G648D substitution. This substitution aligned functionally to a *UPC2* dominant allele previously obtained from *S. cerevisiae* with a G888D substitution (Crowley *et al.*, 1998). The hyperactivity conferred by the *C. albicans UPC2* mutant allele may contribute to increased azole resistance by *ERG11* upregulation, but especially when *ERG11* already contains mutations affecting azole binding.

Because *C. albicans* is diploid, the occurrence of GOF in the *C. albicans* transcription factors *TAC1*, *MRR1* and *UPC2* raises the question of whether these mutations are found in the homozygous or the heterozygous state. Results published with *TAC1* and *MRR1* favor the hypothesis of a so-called codominance effect (Coste *et al.*, 2004, 2007; Dunkel *et al.*, 2008a). This implies that GOF mutations can express their full phenotypes only when found in the homozygous state. In clinical isolates investigated so far, homozygocity at the genomic loci of these genes was achieved by loss of heterozygocity events, which themselves were the results of gene conversions or mitotic recombination events. Further increase of gene copy number can be obtained in *C. albicans* by chromosomal alterations. One of the most spectacular is segmental aneuploidy by isochromosome formation, which was identified for the chromosome 5 left arm (i5L). Interestingly, the chromosome 5 left arm contains *TAC1* and *ERG11*. Therefore, when forming i5L, *C. albicans* not only increase *TAC1* gene copy number with GOF mutations but also increase *ERG11* gene copy number. The association of both factors contributes to increase drug resistance (i.e. azole resistance) to high levels (Selmecki *et al.*, 2006; Coste *et al.*, 2007).

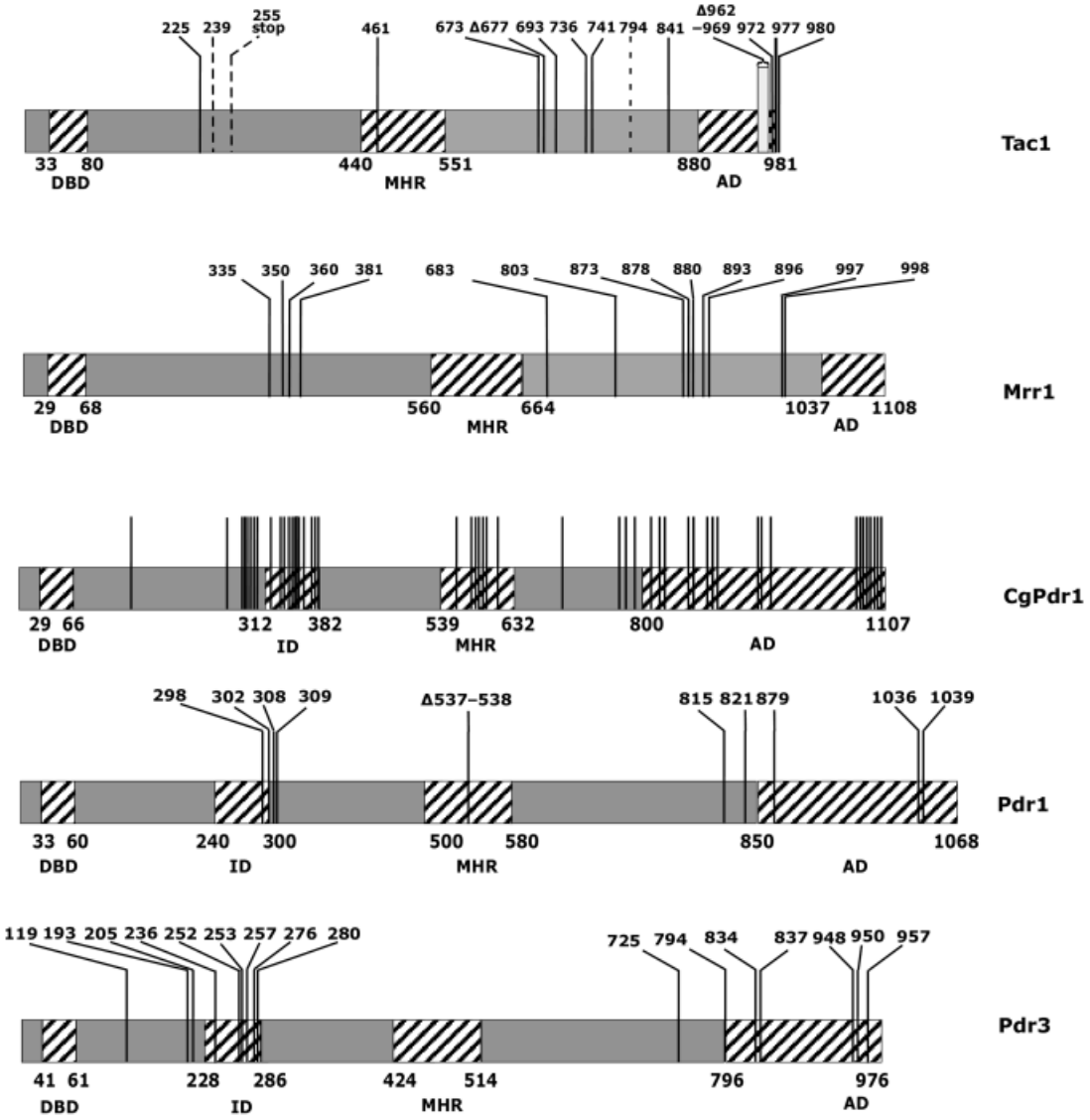

Fig. 3. GOF mutations in transcription factors regulating efflux genes. Repartition of the GOF mutations (black bars) identified in the transcription factors Tac1, Mrr1, CgPdr1, Pdr1 and Pdr3. Hatched bars in Tac1 signify that the GOFs were obtained by random mutagenesis (Coste *et al*., 2009). The GOF mutation deleting amino acid positions 962–969 is indicated by a rectangle. Data for *MRR1*, *CgPDR1*, *PDR1* and *PDR3* were obtained from published reports (Kolaczkowski & Goffeau, 1997; Nourani *et al*., 1997; Kolaczkowska & Goffeau, 1999; Mizoguchi *et al*., 2002; Vermitsky & Edlind, 2004; Morschhauser *et al*., 2007; Dunkel *et al*., 2008a; Ferrari *et al*., 2009). DBD, DNA-binding domain; ID, inhibitory domain; AD, activation domain.

Chromosome alterations as a mode of gene copy increase of drug resistance genes were also recently documented in *C. glabrata*. *De novo* mini-chromosome formation was identified in some azole-resistant isolates. In one case, the mini-chromosome included a genome segment containing *CgCDR2*, an ABC transporter known to contribute to azole resistance (Polakova *et al*., 2009). Although the relevance of this specific effect was not demonstrated by genetic approaches, it suggests that *C. glabrata* is also able to adapt to drug resistance by chromosomal rearrangements.

Genome-wide analysis for the elucidation of transcriptional circuits of drug resistance

The transcriptional regulators discovered for their involvement in the regulation of drug resistance genes may also extend their regulation to other genes as well to other regulators. These genes may eventually establish regulatory connections and together build regulatory circuits. The repertoire of genes regulated by a given transcription factor

can be identified by genome-wide transcript profiling. Together with transcriptome analysis, primary transcription targets can be obtained by chromatin immunoprecipitation (ChIP) using tagged factors and whole genome microarrays (CHIP), a so-called ChIP/CHIP analysis. Such combined analysis may yield regulons of selected transcription factors.

Along with this whole genome rationale, transcriptional circuits responsible for drug resistance could be uncovered by systematic deletion of transcription factor genes. Mutants exposed to a given agent and lacking a wild-type response can be identified in phenotypic screenings. Transcription factor genes identified by this method can reveal additional components of the regulatory circuits responsible for drug resistance. Here, we will illustrate these different approaches by published examples in *C. albicans*.

Transcriptional analysis and whole genome scanning of transcription factor-binding sites

Several groups have exploited microarray analysis to investigate the transcriptional regulation of drug resistance in *C. albicans*. Transcript profiling experiments have been carried out with azole-susceptible and azole-resistant matched isolates from different origins (Rogers & Barker, 2002, 2003; Karababa *et al.*, 2004), with strains having acquired stable azole resistance upon serial passages on fluconazole (Cowen *et al.*, 2002), or with strains with *in vitro* acquired resistance to amphotericin B (Barker *et al.*, 2004). Likewise, the transcriptional response of fungal pathogens to antifungal agents was obtained for the identification of the drug mode of action and associated target genes and the exploration of specific regulatory circuits. This was accomplished by exposing *C. albicans* to azoles, polyenes, 5-FC and echinocandins (Liu *et al.*, 2005) or to ciclopirox (Lee *et al.*, 2005). Taken together, these studies revealed the existence of several regulatory circuits behind the development of antifungal resistance. Analysis of genes differentially expressed between azole-susceptible and azole-resistant isolates from three separate studies and previously known to upregulate *CDR* genes (Cowen *et al.*, 2002; Rogers & Barker, 2003; Karababa *et al.*, 2004) revealed coregulated genes, among them *RTA3* (putative phospholipid flippase), *IFU5* (gene of unknown function) and *GPX1* (putative glutathione peroxidase). These genes were also among those upregulated when *C. albicans* is exposed to fluphenazine (Karababa *et al.*, 2004), a condition previously known to induce both *CDR1* and *CDR2* (De Micheli *et al.*, 2002). Mutants lacking *TAC1* exposed to fluphenazine could not regulate a similar group of genes (i.e. *CDR1*, *CDR2*, *IFU5* and *RTA3*) (Coste *et al.*, 2004), and thus highlights the role of this transcription factor in this type of regulation. As mentioned above, the further characterization of transcription factor regulons requires whole genome scanning of transcription factor-binding sites by ChIP/CHIP analysis. This was accomplished by Liu *et al.* (2007) with a tagged version of Tac1. This landmark study identified, from a set of azole-resistant isolates, 31 genes that were consistently upregulated with *CDR1* and *CDR2*, including *TAC1* itself, and 12 consistently downregulated genes. The genome-wide location of Tac1 by ChIP–CHIP identified 37 genes whose promoters were bound by Tac1 *in vivo*, including *CDR1* and *CDR2*. Interestingly, there were eight genes whose expression was modulated in four azole-resistant clinical isolates in a *TAC1*-dependent manner and whose promoters were bound by Tac1, including *CDR1*, *CDR2*, *GPX1*, *LCB4* (putative sphingosine kinase), *RTA3* and orf19.1887 (putative lipase), as well as *IFU5* and orf19.4898 of unknown function. These genes therefore constitute the core *TAC1* regulon. Sequence analysis in the promoters of these genes identified the consensus [CGGN(4)CGG] as Tac1 drug-responsive element (Liu *et al.*, 2007). This consensus largely agrees with the minimal DRE functional requirements delimited in a recent study (5′-CGGAWATCGGA-TATTTTTTT-3′) (Coste *et al.*, 2009).

Transcript profiling of genes differentially expressed in clinical strains known to upregulate *MDR1* also revealed another cluster of coordinately upregulated genes (Cowen *et al.*, 2002; Rogers & Barker, 2003; Karababa *et al.*, 2004). Besides *MDR1*, these genes are *GRP2*, *IFD5*, orf19.7306 and *SNZ1*. In addition to the antifungal drug resistance function of *MDR1*, the other genes have oxido-reductive functions (*GRP2*, *IFD5*) or are potentially involved in pyridoxine (vitamin B6) synthesis (orf19.7306 and *SNZ1*). As in the case of fluphenazine and *CDR* genes, benomyl exposure can result in the upregulation of *MDR1*. Transcript profiling experiments of cells exposed to benomyl compared with those of clinical strains upregulating *MDR1* revealed a set of coregulated genes, most of them involved in stress response. A group of these genes (*IFD1*, *IFD4*, *IFD5*, *IFD7*) belongs to a family encoding proteins with similarity to putative aryl-alcohol dehydrogenases. With the identification of Mrr1 as a transcriptional regulator of *MDR1*, it has been possible to identify Mrr1-dependent genes in *C. albicans*. Morschhauser *et al.* (2007) proposed a core set of 14 *MRR1*-dependent genes (*MDR1*, orf19.251, orf19.1449, orf19.7166, orf19.6957.3, *IFD1*, *IFD5*, *IFD4*, *IFD6*, *IFD7* orf19.271, orf19.7306, *OYE3* and *GRP2*, the latter nine genes with putative oxido-reductase activities) that were deduced from lists of genes coregulated with *MDR1* in clinical isolates, from genes regulated by *MRR1* GOF mutations or from genes downregulated in *MRR1* null mutant strains. It was striking that most of the genes identified by Morschhauser *et al.* (2007) were also those identified in the above-mentioned studies that investigated *MDR1*-overexpressing strains and benomyl-treated cells. The whole genome occupancy of Mrr1 is still under investigation (J. Morschhauser,

pers. commun.), but will help to precisely elaborate the extent of the Mrr1 regulon.

It is interesting that many Mrr1-dependent genes also belong to a group of genes induced in the presence of H_2O_2, thus showing a convergence between Mrr1-dependent genes and stress response (Enjalbert *et al.*, 2003). Moreover, some of the genes upregulated by H_2O_2 (including *IFD1*, *GRP2*, *IFD5*, *IFD4*, *IFD7*, *GRP4*, orf19.3121, *IFR2*, *TTR1*, orf19.1162, *PST2* and orf19.5517) are those induced by benomyl. Most of these genes are implicated in response to stress or have oxido-reductive functions. Among these, *GRP2*, *GRP4*, orf19.3121, *TTR1*, *PST2* and orf19.5517 contain a putative Cap1-binding site in their promoters. Therefore, a functional linkage involving Cap1 probably exists between benomyl, H_2O_2 exposure and Mrr1-dependent genes. Interestingly, it has been reported in animals that benomyl treatment can cause lipid peroxidation and glutathione depletion. These effects were counteracted with antioxidants, thus probably suggesting that benomyl toxicity could be associated with oxidative stress (Rajeswary *et al.*, 2007). Whether or not this effect applies to yeast is hypothetical.

In *S. cerevisiae*, the Cap1 functional homologue, Yap1, is localized in the cytoplasm and it is only after its activation by oxidative stress that this factor migrates to the nucleus and activates the transcription of genes with Yap1-binding sites in their promoters (Kuge *et al.*, 2001). A similar situation exists in *C. albicans*, as shown by studies carried out by Zhang *et al.* (2000). Genome-wide location of Cap1 was recently performed with ChIP/CHIP (Znaidi *et al.*, 2009). A triple-hemagglutinin (HA_3) epitope was introduced at the C-terminus of wild-type Cap1 (Cap1-HA_3) or hyperactive Cap1 with an altered cysteine-rich domain (Cap1-CSE-HA_3). Location profiling identified 89 targets bound by Cap1-HA_3 or Cap1-CSE-HA_3. Cap1 targets included genes involved in the oxidative stress response (important among them were *CAP1*, *GLR1*, *TRX1*, *SOD1* and *CAT1*), genes involved in response to drugs (important among them were *PDR16*, *MDR1*, *FLU1*, *YCF1* and *FCR1*), genes involved in phospholipid transport (*PDR16*, *GIT1*, *RTA2* and orf19.932) and a few genes involved in the regulation of nitrogen utilization, suggesting that Cap1 has other cellular functions in addition to the oxidative stress response. The authors also identified a good overlap between Cap1-bound genes and those that responded to benomyl, thus reinforcing the hypothesis that stress under benomyl and oxidative conditions converges to the same Cap1-dependent pathway. Bioinformatic analyses of the bound sequences suggest that Cap1 recognizes the DNA motif 5′-MTKASTMA.

Whole genome scanning of Upc2-binding sites was also performed using similar technologies as those used for Tac1 and Cap1. A triple HA epitope, introduced at the C-terminus of Upc2, conferred a GOF effect on the fusion protein. Location profiling identified 202 bound promoters (Znaidi *et al.*, 2008). As expected, genes involved in ergosterol biosynthesis were among those identified by ChIP/CHIP and included *ERG10*, *ERG251*, *ERG2, ERG5*, *ERG11*, *ERG9*, *ERG25*, *NCP1*, *ERG6*, *ERG1*, *ERG24*, *ERG4* and *UPC2* itself. Other relevant enriched functional groups included genes encoding ribosomal subunits, genes encoding drug transporters (*CDR1*, *MDR1* and *YOR1*) and genes encoding transcription factors (*INO2*, *ACE2*, *SUT1* and *UPC2*). Bioinformatic analyses suggested that Upc2 binds to the DNA motif (5′-VNCGBDTR-3′) that includes the previously characterized Upc2-binding site (5′-TCGTATA-3′). In the study published by Znaidi *et al.* (2008), *ERG11*, *MDR1* and *CDR1* transcripts were investigated in wild-type and *upc2*Δ/Δ strains grown under *UPC2*-activating conditions (lovastatin treatment and hypoxia). The authors showed that Upc2 could regulate target genes acting either as an activator or as a repressor depending on the target and the activating condition. For example, *MDR1* expression in the presence of lovastatin was slightly induced in the wild-type strain, but was strongly induced in the *upc2*Δ/Δ mutant. *MDR1* expression in the wild type under hypoxic conditions was slightly induced as compared with normal conditions; however, this induction was abolished in the *upc2*Δ/Δ mutant. This illustrates that Upc2 belongs to a complex regulatory network, which could probably interfere with other transcriptional activators of drug resistance genes.

NDT80 was identified as a *CDR1* regulator and a recent study undertook the genome-wide localization of Ndt80-binding sites (Sellam *et al.*, 2009a). Ndt80 was found to bind a large number of gene promoters (23% of the *C. albicans* genes) with diverse biological functions. Gene ontology analysis of Ndt80 targets revealed a significant enrichment in gene products related to cell wall, carbohydrate metabolism, stress responses, hyphal development, multidrug transport and cell cycle. A surprising number of Ndt80 transcriptional regulators include regulators of hyphal growth (*EFG1*, *NRG1*, *UME6*, *TEC1*, *CPH2*, *FLO8*, *CZF1*, *SSN6*, *RFG1*), carbohydrate metabolism (*RGT1*, *TYE7*, *GAL4*, *MIG1*), cell cycle (*SWI4*, *ASH1*), lipid metabolism (*INO2*, *OPI1*, *CTF1*), translation and amino acid metabolism (*CBF1*, *GLN3*, *GCN4*), stress (*CAT8*, *HAC1*, *CAS5*) and general transcriptional regulators (*SUA71*, *TBP1*, *STP1*, *STP2P*, *STP3*, *STP4*). Promoters of multidrug transporter genes (*CDR1*, *CDR2*, *CDR4* and orf19.4531) were significantly targeted by Ndt80. Furthermore, Ndt80 bound to promoter regions of MFS drug transporters such as *MDR1* and *FLU1*, as well as the two lipid flippases *RTA3* and *RTA2*. Ndt80 target genes also included other *C. albicans* drug resistance genes such as *PDR16* and *ERG3*. Ndt80 also binds a number of other *ERG* genes including the

azole target *ERG11*. The Ndt80-binding motif was deduced as 5′-NaCacAAAa-3′ (where lower-case letters indicate semi-conserved residues and N indicates any nucleotide). The study published by Sellam *et al.* (2009b) included expression profiling to identify fluconazole-responsive genes that require Ndt80 for expression. Interestingly, Ndt80 was crucial for the expression of *ERG* genes including *ERG2*, *ERG25*, *ERG4*, *ERG24*, *ERG13*, *ERG9*, *ERG3*, *ERG10*, *ERG251*, *ERG1*, *ERG5*, *ERG6*, *ERG7*, *ERG11* and *ERG26*. Therefore, because Ndt80 modulates sterol metabolism and drug resistance in *C. albicans*, it represents a major element in the drug response of this yeast species.

The genome-wide location of general components of the transcriptional machinery also revealed an association with drug resistance in *C. albicans*. For example, the genome-wide occupancy of *ADA2* encoding a histone deacetylase, which is part of the SAGA coactivator complex, identified several drug resistance genes as Ada2 targets. These genes include the transporters *MDR1*, *CDR1*, *CDR4*, *QDR1*, *YCF1*, *FLU1*, orf19.4531 and orf19.301 as well as the phosphatidylinositol transfer gene *PDR16*. Most probably, transcription factors controlling the expression of at least *MDR1*, *CDR1* and *PDR16* recruit Ada2 for functional transcription. Sellam *et al.* (2009a) showed that Ada2 occupancy on *MDR1* was completely dependent on Mrr1, demonstrating that Ada2 functions as a coactivator for Mrr1. In addition, transcriptome analysis of an *ADA2* null mutant showed that Ada2 was required for the response to oxidative stress, as well as to treatments with tunicamycin, thus showing that *ADA2* plays an important role in response to stress. It is, however, clear that this activator may play a more global role in transcriptional regulation, because it is likely to assist several other transcription factors. This was well established for the SAGA/ADA coactivator complex in *S. cerevisiae* (Sellam *et al.*, 2009).

It is evident that many additional studies will address the genome-wide occupancy of other transcriptional regulators of drug resistance. *FCR1* and *MRR1* are currently under investigation (M. Raymond and J. Morschhauser, pers. commun.). The genome-wide occupancy of Fcr1p established binding to promoters of drug resistance genes (*ERG11*, *UPC2*), but also genes involved in the transport of small molecules (ammonium, oligopeptides) such as *MEP1*, *CAN1* or *OPT1* and *OPT4* and genes involved in nitrogen utilization (*GAT1*, *SPT3*) (M. Raymond, pers. commun.). Because it regulates *ERG11* and *UPC2*, *FCR1* may be considered as a regulator of azole resistance. The absence of binding to the *CDR1* promoter raises the validity of previous speculations on the role of *FCR1* in *CDR1* regulation (Shen *et al.*, 2007).

As we indicated previously, genome-wide occupancy studies are best exploited when combined with transcriptional analysis. These powerful studies can also aid the elaboration of transcriptional circuits by connecting several transcription factors and establishing a hierarchy in their regulation. In Fig. 4, regulatory circuits modulating the expression of drug target genes (*CDR1*, *MDR1*, *ERG11*) existing in *C. albicans* are schematized. The connections between transcription factors were inferred from existing data published with genome-wide transcript profiling and ChIP assays. Besides the observation that each major transcriptional activator is auto-regulated, it is evident from this figure that even if major transcriptional activators such as *TAC1*, *MRR1* or *UPC2* control each specific subset of genes, cross-talk can exist between their target genes. For example, both *TAC1* and *UPC2* regulate *CDR1*. The same transcription factor can also regulate genes found in separate regulons: for example, *CAP1* regulates both *PDR16* and *MDR1*. Transcription factors can target other transcription factors, which themselves are associated with other

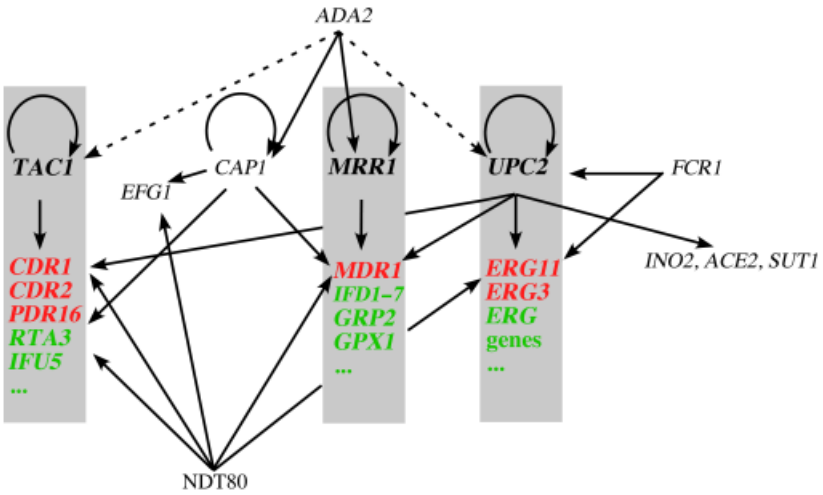

Fig. 4. Regulatory circuits of drug resistance genes in *Candida albicans*. Gene symbols in red signify drug resistance genes; gene symbols in green correspond to genes regulated by a given transcription factor. Gray boxes indicate the presence of a known regulatory pathway. Arrows indicate functional association as deduced from transcript profiling experiments and whole genome occupancy of given transcription factors. Hatched arrows indicate possible (but not proven) interactions. Gene symbols in boldface indicate the presence of GOF mutations obtained from the analysis of clinical strains. See text for other details.

transcriptional units. For example, *EFG1*, which is known as a key regulator of morphogenesis (Noffz *et al.*, 2008), is targeted by both *CAP1* and *NDT80*. This latter transcription factor is intriguingly interacting with several genes belonging to other regulons. As we indicated earlier, *NDT80* is also associated with a wide range of genes with different functions. *ADA2*, which is part of the SAGA coactivator complex, participates in the regulation of drug resistance genes by interacting directly with *MRR1* and *CAP1*, but may also be coactivators of other important factors such as *TAC1* and *UPC2*. Even though the entire repertoire of interactions remains to be discovered, the existing data already suggest complex relationships.

Alternative genomic tools in the discovery of transcriptional regulators of drug resistance

Genome-wide screens of mutants have been undertaken in yeast pathogens including *C. albicans* and *C. glabrata* for the identification of genes involved in drug resistance (Bruno & Mitchell, 2004; Kaur *et al.*, 2004; Bruno *et al.*, 2006; Rauceo *et al.*, 2008). In *C. albicans*, mutants have been generated by a random collection of DNA fragments interrupted by transposon mutagenesis in *E. coli*. In this strategy, the DNA fragments containing interrupted ORF were transformed into *C. albicans* and homozygous mutants selected by a two-step selection procedure (Davis *et al.*, 2002).

Mutant collections covering different gene families (transcription factors, kinases, cell wall proteins) have been screened as of now using azoles or candins as major antifungal agents. Genes critical for the wild-type response to both agents were isolated, including *CAS5*, *ADA2*, *SKO1* and *CKA2*. *CAS5* belongs to the cluster of Zn_2-Cys_6 transcription factors and *ADA2* is a transcriptional coactivator. Inactivation of both genes resulted in increased candin susceptibility (Bruno *et al.*, 2006). Because Ada2 is required for expression of many Cas5-dependent genes, it is likely that Cas5 recruits Ada2 to activate target gene transcription (Bruno *et al.*, 2006). The *SKO1* defect also caused increased susceptibility to candins. This transcription factor undergoes phosphorylation in *S. cerevisiae* in order to migrate to the nucleus and activate the transcription of target genes (Proft *et al.*, 2001). As in *S. cerevisiae*, the *C. albicans* Sko1p undergoes Hog1-dependent phosphorylation after osmotic stress, thus showing a dependence of Sko1 on Hog1. However, Sko1 does not undergo detectable phosphorylation in response to caspofungin and thus Sko1-dependent gene expression depends on additional factors (Rauceo *et al.*, 2008). The same study identified, from a panel of caspofungin-hypersensitive protein kinase-defective mutants, the protein kinase Psk1 as required for expression of *SKO1* and of Sko1-dependent genes in response to caspofungin. The connection between Psk1 and Sko1 highlights a novel signal transduction pathway that modulates the expression of genes necessary for the wild-type response to caspofungin. *CKA2* showed similarity to the *S. cerevisiae CKA2* encoding casein kinase II. In a *cka2* mutant, *CDR1* was upregulated, thus explaining the decreased susceptibility to fluconazole. Interestingly, expression of *RTA3*, a gene previously identified as upregulated in an azole-resistant strain (Karababa *et al.*, 2004) and also dependent on the transcription factor *TAC1* (Coste *et al.*, 2006), was also dependent on the inactivation of *CKA2*. *CKA2* is therefore a negative regulator of *CDR1* and could operate at still unknown levels with *TAC1* in a common pathway of activation (Bruno & Mitchell, 2005).

In *C. glabrata*, strategies to establish random collection of mutants are facilitated by the haploidy of this yeast. Transposon mutagenesis has been directly followed by transformation of transposon-interrupted DNA fragments into *C. glabrata*. Mutant collections were thus generated, containing a wide range of mutants (Castano *et al.*, 2003). A mutant collection (9216 random insertion mutants) was screened for fluconazole susceptibility and several mutants (27) were identified (Kaur *et al.*, 2004). Homologues of three of these genes have been implicated in azole and/or drug resistance in *S. cerevisiae*: two of these belong to the family of ABC transporters and phosphatidylinositol transporter (*PDR5* and *PDR16*), and one is involved in retrograde signaling from the mitochondria to the nucleus (*RTG2*). The remaining 24 genes were involved in diverse cellular functions, including ribosomal biogenesis and mitochondrial function, activation of RNA polymerase II transcription, nuclear ubiquitin ligase function, cell wall biosynthesis and calcium homeostasis. The involvement of these biological functions in the response of *C. glabrata* to fluconazole underscores the extent of alterations resulting from the exposure to an antifungal agent (Kaur *et al.*, 2004).

Conclusions and perspectives

In this review, we summarized the present knowledge on the transcriptional regulation of drug resistance genes in several fungal species, although most knowledge has been assembled in *C. albicans*. The recent work accomplished by several groups has enabled the identification of core transcription factors involved in the regulation of major players of drug resistance including the *C. albicans TAC1*, *MRR1* and *UPC2* genes or *CgPDR1* in *C. glabrata*. Their relevance in the development of resistance in clinical isolates has been demonstrated by the identification of several mutations. Accessory factors including *ADA2*, *NDT80*, *FCR1* or *REP1* important for modulating *CDR1* or *MDR1* expression have been added to these core elements. Combined with studies constituting the fundamentals of our understanding in the development of drug resistance, the use of genome-wide

approaches has now started to reveal the complexity of regulatory circuits controlling drug resistance. Genome-wide genetic screens for the identification of factors involved in drug resistance or participating in the response of fungal pathogens to drug exposure will likely result in the identification of additional players in this process. Furthermore, because transcription factors cooperate with coactivators necessary for the recruitment of the transcriptional machinery, it will be necessary to understand the specific requirements of important transcription factors for these elements.

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