

What ‘Omics can tell us about antifungal adaptation

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One sentence summary: ‘Omics technologies provide vital insights into the physiological adaptations that occur in response to antifungals, but key gaps remain in understanding antifungal adaptation in model yeast and pathogenic fungi.

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ABSTRACT

Invasive candidiasis, the most frequent healthcare-associated invasive fungal infection, is commonly caused by *Candida albicans*. However, in recent years other antifungal-resistant *Candida* species—namely *Candida glabrata* and *Candida auris*—have emerged as a serious matter of concern. Much of our understanding of the mechanisms regulating antifungal resistance and tolerance relies on studies utilizing *C. albicans*, *C. glabrata* and the model yeast *Saccharomyces cerevisiae*. ‘Omics studies have been used to describe alterations in metabolic, genomic and transcriptomic expression profiles upon antifungal treatment of fungal cells. The physiological changes identified by these approaches could significantly affect fungal fitness in the host and survival during antifungal challenge, as well as provide further understanding of clinical resistance. Thus, this review aims to comparatively address ‘omics data for *C. albicans*, *C. glabrata* and *S. cerevisiae* published from 2000 to 2021 to identify what these technologies can tell us regarding cellular responses to antifungal therapy. We will also highlight possible effects on pathogen survival and identify future avenues for antifungal research.

Keywords: ‘Omics, *Candida albicans*, *Candida glabrata*, *Saccharomyces cerevisiae*, antifungals, resistance

INTRODUCTION

Invasive candidiasis is a life-threatening fungal disease that is most often caused by *Candida albicans*. The incidence of invasive candidiasis is estimated at ~5100 individuals per year in the UK and ~25 000 patients per year in the USA, and in particular affects patients on intensive chemotherapy, immunosuppressive drugs or long-term hospital stays (Bongomin et al. 2017; Pegorie, Denning and Welfare 2017; Tsay et al. 2020). However, recent increases in the clinical incidence of other non-*albicans* species have been observed, particularly for antifungal-resistant species (Guinea 2014; Lamoth et al. 2018; Ricotta et al. 2020). This increasing frequency of infections by drug and multi-drug resistant fungal pathogens presents a complex modern clinical challenge that requires urgent attention.

Candida glabrata and *Candida auris* are two important species of concern that can cause drug-resistant candidiasis. *Candida glabrata* is the second leading cause of invasive candidiasis in several geographical regions, including North America and Europe (Bongomin et al. 2017; Ricotta et al. 2020). *Candida glabrata* rapidly acquires resistance to azoles and can develop resistance to a second drug class, the echinocandins (Healey and Perlin 2018). *Candida auris* is an emerging fungal pathogen that is classified as a serious global health threat by the Centers for Disease Control due to its alarming rates of multi-drug resistance, with some isolates resistant to all three major antifungal drug classes (CDC 2021). Since its first description in 2009 (Satoh et al. 2009), *C. auris* has caused multiple hospital outbreaks prompting calls for improved diagnostics and renewed efforts for antifungal development. However, we currently do not know how antifungal resis-

tance mechanisms exhibited by these species affect transmission, commensalism and other aspects of host–pathogen interactions.

Drug susceptibility can be quantified *in vitro* by minimum inhibitory concentration (MIC) testing. Antifungal resistance is usually defined as the acquisition of genetic mutations within a population that confers the ability to grow at high MICs. Antifungal tolerance permits growth in the zone of inhibition, but is distinct from resistance. Tolerant sub-populations can proliferate slowly at high azole MICs and survive lethal echinocandin challenge without acquiring adaptive genetic mutations or altering the MIC of the population (Robbins, Caplan and Cowen 2017; Healey and Perlin 2018; Rosenberg et al. 2018). However, little is known about the mechanisms that drive tolerant adaptation versus antifungal resistance.

Much of our current understanding of the molecular mechanisms underlying antifungal resistance or tolerance in *Candida* spp. relies on work undertaken in *C. albicans*, *C. glabrata* and the model yeast, *Saccharomyces cerevisiae*. Interestingly, many antifungal resistance mechanisms are conserved between these organisms, and these will be discussed further in this review. In addition to these canonical antifungal resistance mechanisms, ‘omics studies have highlighted massive changes in metabolic flux and gene and protein expression profiles when cells are stressed by antifungals (Figs 1 and 2). These alterations in physiological processes are poorly understood, but could ultimately affect host fitness and survival during antifungal challenge and provide greater insight into clinical resistance. Therefore, this review will address what ‘omics data from *S. cerevisiae*, *C. albicans* and *C. glabrata* can tell us about cellular responses to antifungal therapy and high-

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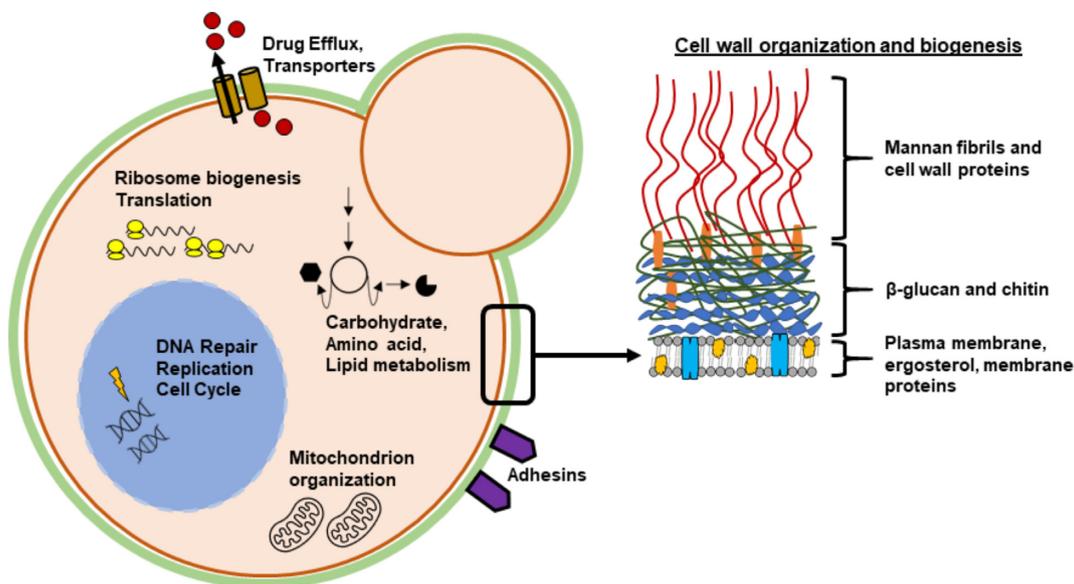


Figure 1. Common biological processes affected by antifungal treatment. This yeast cell diagram highlights GO Biological Processes that were frequently differentially regulated in antifungal 'omics studies.

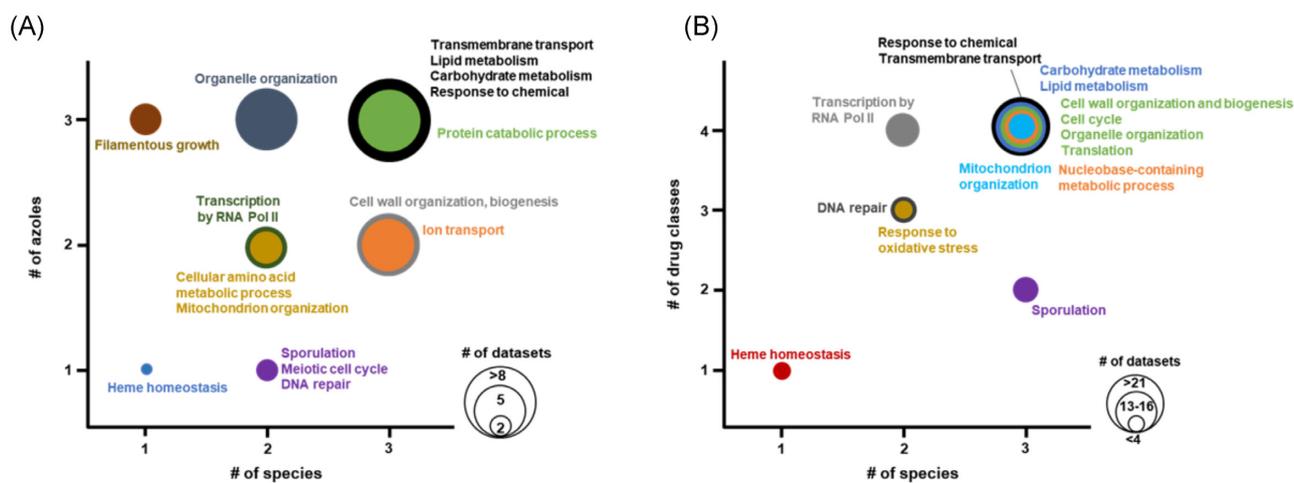


Figure 2. Bubble chart for the top recurrent GO Biological Process terms across antifungal 'omics datasets. Publicly available datasets from the articles listed in supplemental data were analyzed for GO Slim Biological Processes on SGD and CGD. Bubble size scales with the number of datasets where the GO Biological Process was differentially expressed in antifungal treated versus control cells. **(A)** Differentially regulated GO Biological Processes for *S. cerevisiae*, *C. glabrata* and *C. albicans* transcriptomic and proteomic datasets featuring fluconazole, miconazole or ketoconazole treated cells. **(B)** A subset of the top differentially regulated GO Biological Processes for *S. cerevisiae*, *C. glabrata* and *C. albicans* transcriptomic, and proteomic datasets for all four drug classes (i.e. 5-flucytosine, amphotericin B, caspofungin and azole drugs). Note: panel B bubble scale is determined by the number of datasets in multiples of 4 (i.e. 1–4, 5–8, 9–12, 13–16, 17–20 and 21–24 datasets).

light how these responses may affect pathogen survival during commensalism or infection.

OVERVIEW OF MOLECULAR ANTIFUNGAL RESISTANCE MECHANISMS

Clinical resistance is defined as infection persistence due to a failure to inactivate or kill fungal pathogens despite appropriate treatment (Kanafani and Perfect 2008). Clinical resistance is not always correlated with *in vitro* resistance, which is often measured as the MIC of a given drug. There is speculation that this lack in correlation is due to, perhaps in part, the multivariate nature of clinical resistance, which relies on the interaction between the pathogen, the host and the pharmacokinetics of the chosen drug.

The antifungal drugs currently used in the clinic for invasive disease can be divided into four classes based on their mechanism of action: azoles, echinocandins, polyenes and flucytosine (5-flucytosine, 5-fluorocytosine, or 5-FC). Major mechanisms for resistance to these drugs are listed in Table 1 and briefly summarized below (and addressed in more detail in these reviews; Sanglard, Coste and Ferrari (2009), Cowen et al. (2014) and Bhattacharya, Sae-Tia and Fries (2020)). Treatment options for fungal infections remain limited largely because many compounds, although effective, are extremely toxic to mammalian cells due to similarities between host and fungal cell biology. These therapeutic limitations underscore the risks of emerging antifungal resistance and the need for continued development of new antifungals.

Table 1. Key mechanisms and genes involved in antifungal resistance.

	<i>S. cerevisiae</i>	<i>C. glabrata</i>	<i>C. albicans</i>
Azoles			
Ergosterol biosynthesis upregulation via	✓	✓	✓
• <i>ERG3/6</i> loss of function mutation	<i>ERG3/6/11</i>	<i>ERG11</i>	<i>ERG3/11</i>
• <i>ERG11</i> or <i>UPC2</i> gain-of-function mutations or overexpression	<i>UPC2</i>	<i>UPC2A</i>	<i>UPC2</i>
Increased efflux pump activity via	✓	✓	✓
• <i>PDR1</i> , <i>MRR1</i> or <i>TAC1</i> gain-of-function mutations	<i>PDR1</i>	<i>PDR1</i>	<i>TAC1</i> and <i>MRR1</i>
• <i>CDR1/2</i> overexpression	<i>PDR5</i>	<i>CDR1/2</i>	<i>CDR1/2</i>
Increased ABC transporter activity via	✓	✓	✓
• <i>STB5</i> loss of function mutation		<i>STB5</i>	<i>STB5</i>
• <i>SNQ2</i> , <i>PDH1</i> or <i>YOR1</i> overexpression	<i>SNQ2</i>	<i>SNQ2</i> and <i>PDH1</i> , <i>YOR1</i>	<i>YOR1</i>
Increased drug:H + antiporter activity via	✓	✓	✓
<i>AQR1</i> , <i>FLR1</i> , <i>QDR2</i> , <i>TPO1_1</i> or <i>MDR1</i> overexpression	<i>AQR1</i> and <i>FLR1</i>	<i>QDR2</i> and <i>TPO1</i>	<i>MDR1</i>
Echinocandins			
Modification of the glucan synthase enzyme or its activity	✓		
• <i>FKS1</i> and <i>FKS2</i> mutations	<i>FKS1/2</i>	<i>FKS1/2</i>	<i>FKS1/2</i>
• <i>SBE2</i> overexpression	<i>SBE2</i>		
Long chain base accumulation (<i>CRIS-MIS</i>), <i>SUR2</i> and <i>FEN1</i> loss of function	✓	✓	✓
Disturbance in programmed cell death, <i>AIF1</i> loss of function mutation	✓	✓	✓
Increased chitin levels (<i>CHS</i> mutations)	✓	✓	✓
Polyenes			
Depleted ergosterol from the cell membrane, <i>ERG1/2/3/5/6/11</i> loss of function	✓	✓	✓
	<i>ERG3/6</i>	<i>ERG1/2/6/11</i>	<i>ERG3/5</i>
Reduced ribosome synthesis, <i>TORC1</i> loss of function	✓	✓	✓
Increased reactive oxygen species	✓	✓	✓
Detoxification, <i>SOD3</i> , <i>RAS1/2</i> , <i>TOR1</i> or <i>BSC2</i> mutations	<i>RAS1/2</i> , <i>TOR1</i> and <i>BSC2</i>		<i>SOD3</i>
5-FC			
Decreased enzyme activity preventing the conversion chain of 5-FC into fungistatic 5-FUTP, <i>FUR1</i> , <i>FCY1/2</i> and <i>FCY21/22</i> loss of function mutation	<i>FUR1</i> and <i>FCY1/2</i>	<i>FUR1</i> and <i>FCY1/2</i>	<i>FUR1</i> and <i>FCY1/2</i>
	<i>FCY21/22</i>		<i>FCY22</i>
Continuation of DNA synthesis despite the presence of fluorinated analogues, <i>CDC21</i> overexpression	✓	✓	✓
Prevention of drug accumulation by hyperactive transporters or antiporters, <i>FLR1/2</i> , <i>CDR1</i> and <i>PDR1</i>	✓	✓	✓

Several antifungal resistance mechanisms are conserved in *S. cerevisiae*, *C. glabrata* and *C. albicans*. Species-specific genes and processes are indicated where appropriate.

Azoles

Candida spp. azole resistance became a clinical concern shortly after the market debut of fluconazole in the late 1980s (Smith et al. 1986). Azoles can be subdivided based on their chemical structures into imidazoles (e.g. ketoconazole and miconazole) and triazoles (e.g. fluconazole, itraconazole and voriconazole). Azole-resistant isolates from candidemia patients have been observed at low frequency for *C. albicans* infections (0–5%), but are frequently found in *C. glabrata* infections (11–15%; Diekema et al. 2012; Pfaller, Jones and Castanheira 2014; Pfaller et al. 2015). Azoles target and inhibit a key enzyme in the ergosterol biosynthetic pathway, lanosterol 14 α -demethylase (*ERG11* in *Candida* spp.). Ergosterol is the major fungal sterol present in the plasma membrane and contributes to the permeability and fluidity of the membrane, ensures

cytoskeleton organization and regulates the activity of membrane transporters (Sgherri et al. 2014).

Azole resistance in *S. cerevisiae*, *C. glabrata* and *C. albicans* has been linked *in vitro* to two general mechanisms—alterations in sterol metabolism or reducing intracellular drug concentrations. First, gain-of-function (GOF) mutations or alterations in the expression of genes linked to ergosterol biosynthesis, such as the azole target *ERG11* (Kontoyiannis, Sagar and Hirschi 1999; Hull et al. 2012a), *ERG6* (Anderson et al. 2003; Xu et al. 2007), *ERG3* (Anderson et al. 2003; Martel et al. 2010), or the sterol metabolism transcriptional regulator *UPC2* (Dunkel et al. 2008a; Whaley et al. 2014), can render cells less sensitive to azole activity. Loss of function mutations in *ERG3* initiate a metabolic bypass that prevents the accumulation of toxic sterol intermediates, which renders cells

less susceptible to growth inhibition by azoles (Martel et al. 2010). Second, cells can limit cytoplasmic azole concentrations via up-regulation of drug efflux pump expression, such as the ABC transporters CDR1, CDR2 and SNQ2, through pump promoter mutations (Sanglard et al. 1995; Mahé et al. 1996; Torelli et al. 2008) or GOF mutations to the transcription factors PDR1, (*S. cerevisiae* and *C. glabrata*), MRR1 (Dunkel et al. 2008b) or TAC1 (*C. albicans*; Mahé et al. 1996; Coste et al. 2004; Vermitsky and Edlind 2004; Tsai et al. 2006). In addition to drug transporter expression, PDR1 controls expression of RPN4, a transcriptional regulator of proteasomal genes that also mediates azole susceptibility in *S. cerevisiae* (Owsianik, Balzi and Ghislain 2002) and *C. glabrata* (Pais et al. 2020). CgPDR1 also affects the expression of the adhesin EPA1, and PDR1 GOF mutations have been associated with increased adherence to epithelial cells and enhanced virulence in mouse candidemia models (Ferrari et al. 2009; Vale-Silva et al. 2016). *Candida glabrata* clinical isolates are intrinsically less susceptible to azole drugs due to their high-level expression of drug efflux pumps (Vermitsky and Edlind 2004). Mutations in ergosterol biosynthesis genes and alterations in efflux pump expression have also been observed in drug-resistant clinical isolates (vanden Bossche et al. 1992; Marichal et al. 1999; Rogers and Barker 2003; Bennett, Izumikawa and Marr 2004; Xiang et al. 2013).

Echinocandins

The echinocandins are the most recent of the four antifungal drug classes to be developed with caspofungin hitting the market in the early 2000s. Echinocandins (i.e. caspofungin, micafungin and anidulafungin) are the preferred first-line choice for treatment of invasive *Candida* infections, in part due to the increasing prevalence of azole-resistant non-*albicans Candida* species (Pappas et al. 2016). This drug class inhibits β -glucan synthesis leading to a loss of cell wall integrity that can be fungicidal or fungistatic. Approximately 2–3% of *C. albicans* (Castanheira et al. 2010) clinical isolates develop echinocandin resistance compared to 1–10% of *C. glabrata* isolates, depending on the geographical region surveyed (Perlin 2015).

Clinical and *in vitro* echinocandin resistance in *S. cerevisiae*, *C. albicans* and *C. glabrata* is largely conferred by point mutations in the major glucan synthase enzymes, FKS1 and FKS2 (Douglas et al. 1997; Johnson, Katiyar and Edlind 2011; Pham et al. 2014; Suwunakorn et al. 2018). These point mutations interfere with or inhibit echinocandin interactions with glucan synthase. In *C. albicans* and *S. cerevisiae*, alterations in programmed cell death due to mutations in AIF1 also can affect echinocandin resistance (Markovich et al. 2004). In addition, caspofungin-treated *C. albicans* and *fks1Δ S. cerevisiae* strains have increased cell wall chitin content compared to untreated or wild-type cells, respectively (Markovich et al. 2004; Walker, Gow and Munro 2013). However, *C. glabrata* does not alter chitin content during exposure to echinocandins (Walker, Gow and Munro 2013). In *C. glabrata*, loss of SUR2 or FEN1 function alters echinocandin susceptibility by modulating sphingolipid interactions with Fks (Healey et al. 2012). A similar phenotype was described for one *C. albicans* strain out of ten tested, which suggests that this method of generating echinocandin resistance is a low-frequency event in this species (Healey et al. 2015).

Polyenes

Amphotericin B (AmB) was first discovered in 1955 and put to clinical use in 1958 making it one of the oldest of the four drug classes used against invasive fungal disease. AmB binds ergosterol in the plasma membrane leading to pore formation and ultimately cell

death. Clinical resistance to AmB is low for *C. albicans* and *C. glabrata* and a recent multi-site study reported no AmB resistant candidemia isolates (Toda et al. 2019). However, AmB also exerts cytotoxic activity against mammalian cells which can induce organ damage, especially to kidneys (Allen 2010). Mammalian toxicity can be reduced with the use of liposomal formulations (Roberts et al. 2015).

Like resistance to azoles, resistance to AmB has been linked to alterations in ergosterol biosynthesis. In *C. albicans*, *C. glabrata* and *S. cerevisiae*, mutations in ERG genes confer some protection against AmB by depleting ergosterol from the plasma membrane (Geber et al. 1995; Kelly et al. 1996; Sanglard et al. 2003; Vandeputte et al. 2008; Martel et al. 2010; Hull et al. 2012b; Kodedová and Sychrová 2015). In addition, decreased TORC1 function confers some AmB resistance by limiting ribosome synthesis resulting in reduced cell growth rates (Bojsen et al. 2016). Recent work in *C. albicans* suggested that AmB induces cellular oxidative stress that plays a role in cidality (Muzafar et al. 2020). Thus, alterations in SOD3 expression led to increased cell survival after drug treatment by detoxifying intracellular reactive oxygen species (ROS). In *S. cerevisiae*, AmB-resistance was linked with altered expression of RAS1, RAS2 or BSC2, which improved ROS detoxifying activities by enhancing expression of glutathione (Bojsen et al. 2016; Kong et al. 2020). ROS detoxifying activity has not been confirmed as a major mechanism of AmB resistance in *C. glabrata*.

Flucytosine

5-FC has been available since 1957. In fungi, 5-FC is converted by cytosine deaminase into 5-fluorouracil (5-FU), which is incorporated into RNA and other metabolites and ultimately interferes with protein translation and DNA synthesis (Polak and Scholer 1975). While initially effective, resistance to this drug is common when used alone, therefore, 5-FC is predominantly used in combinatorial treatment strategies with the above drug classes. Resistance mechanisms to 5-FC are highly conserved in *C. albicans*, *C. glabrata* and *S. cerevisiae*. All three species have demonstrated resistance with loss of function mutations to FUR1, FCY1 or FCY2, resulting in decreased conversion of 5-FC to 5-FU (Erbs, Exinger and Jund 1997; Dodgson et al. 2004; Paluszynski et al. 2006; Edlind and Katiyar 2010). In addition, overexpression of thymidylate synthase can bypass DNA synthesis inhibition in the presence of drug (Vandeputte et al. 2011). Mutations in FCY21/22, the purine–cytosine permease, in *S. cerevisiae* or *C. albicans* inhibits uptake of 5-FC into the cell (Hope et al. 2004; Paluszynski et al. 2006). Finally, expression of drug efflux pumps and antiporters in *C. glabrata*, including increased expression of FLR1, CDR1 and the transcription factor PDR1, confers some resistance to 5-FC (Steier et al. 2013; Pais et al. 2016a).

Other mechanisms involved in drug resistance

Invasive pathogens have a variety of physiological responses that allow them to adapt to otherwise toxic conditions and thus exhibit mechanisms of resistance to antifungals. First, the formation of biofilms—an association of cells enveloped by extracellular matrix (ECM) which provides protection against the external environment (Uppuluri et al. 2011; Ramage et al. 2012)—reduces antifungal diffusion to fungal cells. Consequently, the MIC values required to inactivate biofilm cells were higher when compared to non-biofilm forming isolates or planktonic-grown cells (Chandra et al. 2001; Mukherjee et al. 2003). In 2001, Chandra et al. demonstrated that as biofilms matured the MIC concentrations for distinct antifungal classes also gradually increased for *C. albicans*,

with MICs for fluconazole and voriconazole increasing by as much as 6-fold when comparing 72 h biofilms with the initial 2 h time point. Besides an intricate ECM-cell interaction, biofilms express higher levels of efflux pumps and exhibit altered metabolic states, which further contributes to reduced drug susceptibility (Chandra et al. 2001; Mukherjee et al. 2003; Ramage et al. 2012). Comparatively, for *S. cerevisiae*, Bojsen, Regenber and Folkesson (2014) observed that the response of developing biofilms to antifungals was similar to the response of exponentially growing cells. This similarity was also observed between mature biofilm and non-growing planktonic yeast cells (Bojsen, Regenber and Folkesson 2014). These results suggest that the effects of antifungals were independent of biofilm or planktonic growth in *S. cerevisiae*. Further, Bojsen et al. (2016) suggested that *C. glabrata* mature biofilm drug susceptibility was similar to *S. cerevisiae*, however a gradual assessment of the response of biofilm or planktonic yeast cells to antifungals was not performed for *C. glabrata*.

In addition, Hsp90 is a molecular chaperone that plays an integral role in echinocandin resistance *in vitro* via its regulatory role in the cell wall integrity pathway. Hsp90 modulates the stability of key members of the Protein Kinase C (PKC) pathway (Leach et al. 2012). In response to cell wall damage, the PKC pathway triggers the phosphorylation of Slt2 (whose respective yeast homolog is Mkc1 in *C. albicans*) which initiates the Mitogen Activated Protein (MAP) kinase signaling cascade to activate downstream targets (Leach et al. 2012). These downstream targets include cell wall-associated genes such as chitin biosynthesis enzymes, whose role in increasing cell wall chitin content correlates with improved fungal survival in response to echinocandin treatment (Reinoso-Martin et al. 2003; Cota et al. 2008; Walker, Gow and Munro 2013). In *S. cerevisiae*, deletion of *SLT2*, *BCK1*, *PKC1* or *FKS1* results in caspofungin hypersensitivity (Reinoso-Martin et al. 2003). Functional genomic screening of two *C. albicans* mutant libraries (covering approximately 45% of the genome) indicated that three of the nine genes identified as being involved in modulating echinocandin resistance and tolerance are components of the PKC cell wall integrity cascade (*PKC1*, *SWI4* and *MKC1*; Caplan et al. 2018). Upon further testing of the *Pkc1*-MAPK pathway, Caplan et al. (2018) observed that Hsp90 is necessary for maintaining the stability of *C. albicans* *Pkc1* and *Bck1*, thus allowing for the development of Hsp90-regulated echinocandin resistance as a possible mechanism to compensate for the altered expression of *FKS1*. This Hsp90-dependent echinocandin resistance, mediated by calcineurin, has also been observed in *C. glabrata* clinical isolates (Singh-Babak et al. 2012). More specifically, in *C. glabrata* caspofungin-induced *FKS2* is dependent on calcineurin and Hsp90, and this mechanism can be pharmacologically inhibited to limit basal tolerance and confer echinocandin susceptibility in clinical isolates. Hsp90 is also important in stabilizing calcineurin in *S. cerevisiae* and enables calcineurin-dependent responses to drug-induced cellular stresses; however, Hsp90 does not appear to modulate echinocandin susceptibility in this yeast (Singh et al. 2009; Singh-Babak et al. 2012).

Candida albicans resistance to the polyene AmB has been linked to Hsp90. However, Vincent et al. (2013) observed that AmB-resistant strains were hypersensitive to Hsp90 inhibitors due to high levels of Hsp90 function in cells even in the absence of AmB. This finding has been speculated to be the result of significant costs to fungal pathogenicity in AmB resistant strains, which includes hypersensitivity to host immune defenses and inability to invade host tissue (Vincent et al. 2013). Therefore, the virulence costs because of reduced susceptibility to AmB seems to lead to an evolutionary impasse, making it unfavorable for fungal cells to

present AmB resistance in the clinic. The relevance of AmB resistance and the involvement of Hsp90/calcineurin in this process for *C. glabrata* and *S. cerevisiae* requires further study.

Finally, mitochondrial alterations and activation of stress pathways are also mechanisms utilized by pathogenic fungi to acquire resistance against antifungal agents. For example, loss of mitochondrial function, such as in petite mutants, in *C. glabrata* leads to increased fluconazole resistance (Sanglard, Ischer and Bille 2001). Petite mutants have elevated expression of drug efflux pumps, such as *PDR5* and *CDR1* (Brun et al. 2004; Demuyser et al. 2017). Overexpression of *MGE1*, a yeast chaperone involved in the mitochondrial protein import system, also suppresses fluconazole susceptibility in *S. cerevisiae* and *Candida* species (Demuyser et al. 2017).

GENOMIC ALTERATIONS INVOLVED IN ANTIFUNGAL RESISTANCE

Besides alterations in cell structure, metabolism and membrane homeostasis, antifungals can induce significant genomic changes in fungal cells. This section will explore what we know about antifungal-induced genomic plasticity in *C. albicans*, *C. glabrata* and *S. cerevisiae*.

The role of mating, aneuploidy and isochromosomes in antifungal adaptation

Mating is a mechanism for generating genetic diversity and can be induced by antifungal stress in *C. albicans* (Rustad et al. 2002). In diploid cells, drug-resistant isolates are, for the most part, homozygous for the genetic mutations selected by drug-related external pressures (Rustad et al. 2002). This is evidenced by the observation that loss of heterozygosity (LOH) in a series of clinical isolates led to selection for an altered 'fluconazole-resistant' allele that enhanced antifungal resistance (Rustad et al. 2002). Fluconazole and other stresses intensify the frequency with which these genomic mutations occur (Rustad et al. 2002; Forche et al. 2011; Harrison et al. 2014). Much of our understanding regarding drug adaptation and mating comes from the *C. albicans* literature. *Candida glabrata* is currently assumed to be asexual (Boisnard et al. 2015), and there is little information about how *S. cerevisiae* sexual reproduction impacts antifungal adaptation.

Typically, heterozygosity of the *MTL* locus in *C. albicans* hinders cells from mating (Rustad et al. 2002; Popp et al. 2019). However, genome rearrangements, including transient aneuploidies, mitotic recombination and whole-chromosome loss or duplication can result in *MTL* homozygosity which, in turn, allows for mating-competency to be achieved (Popp et al. 2019). *MTL* homozygosity is not sufficient to confer fluconazole drug resistance, but homozygosity of other genes, such as *ERG11* and drug efflux pumps, play an important role in this process (Rustad et al. 2002; Pujol et al. 2003; Popp et al. 2019). Mating in *C. albicans* populations usually occurs between cells within a clonal population, which can be used by cells as a mechanism to combine advantageous traits for adaptation and resistance to antifungal drugs. Fluconazole-induced *MTL* homozygous cells can also become homozygous for antifungal resistance mutations (Popp et al. 2019). Popp et al. (2019) observed that fluconazole-induced *MTL* homozygous progeny were mating competent, but the initial mating product of these parental strains did not exhibit higher drug resistance than parent cells until exposed to additional selective pressure. These findings suggest that fluconazole treatment selects for resistance mutations and promotes genomic alterations that confer

mating competence, which can propagate mutations linked with fluconazole resistance (Popp *et al.* 2019).

Azole resistance has also been linked with aneuploidy, which can improve stress resistance by increased gene dosage for key adaptive mechanisms. Azole-resistant aneuploids can be derived from *C. albicans* tetraploids which are formed *in vitro* by fluconazole-induced mitotic collapse (Harrison *et al.* 2014). In addition, an isochromosome formed by a specific segmental aneuploidy of the two left arms of chromosome 5 (Ch5) in *C. albicans* confers azole resistance (Selmecki, Forche and Berman 2006). This resistance strategy provides additional copies of *ERG11* and *TAC1* which encode the azole-targeted enzyme in the ergosterol biosynthetic pathway and a transcription factor that positively regulates ABC transporters involved in azole efflux, respectively (Selmecki, Forche and Berman 2006). More recent work has discovered that caspofungin can induce LOH and changes in DNA content in both diploids and tetraploids of *C. albicans* (Avramovska and Hickman 2019). Interestingly, *C. albicans* genome instability also can be induced with other cell wall perturbing agents, including calcofluor white (Avramovska and Hickman 2019).

While much of what we know concerning aneuploidy and drug resistance stems from *C. albicans* research, a *C. glabrata* isolate is the first known case of aneuploidy linked with clinical azole resistance (vanden Bossche *et al.* 1992). The chromosome encoding *ERG11* was duplicated in its entirety in this clinical isolate. Further, the use of aneuploidy to overcome stress is not restricted to pathogenic fungi. *Saccharomyces cerevisiae* can employ aneuploidy to cope with nutrient limitation and proteotoxic stresses (Mulla, Zhu and Li 2014). However, aneuploidy is a risky adaptive mechanism that is often associated with fitness defects due to either increased gene dosage or LOH of many genes with potentially deleterious mutations.

GOF and other mutations

We briefly discussed above how antifungal resistance can be acquired via key GOF mutations. These mutations typically regulate antifungal susceptibility by altering patterns of target gene expression, with targets including efflux pumps, drug targets or transcriptional regulators of efflux pumps and lipid biosynthesis (such as *CaTAC1*, *Sc/CgPDR1*, *CaMRR1* and *Sc/Cg/CaUPC2*; Dunkel *et al.* 2008b; Morschhauser *et al.* 2007; Lohberger, Coste and Sanglard 2014). Although GOF mutations can be beneficial to cell survival during antifungal exposure, these mutations can potentially affect fungal virulence and fitness in the absence of selective drug pressure.

GOF mutation fitness has been investigated both *in vitro* and *in vivo* for *C. albicans*. Strains carrying hyperactive alleles of *TAC1* (N9777D), *MRR1* (G963S) and *UPC2* (G648D), which confer azole resistance, were assessed for virulence in a systemic murine infection model (Lohberger, Coste and Sanglard 2014). Lohberger, Coste and Sanglard (2014) showed that *TAC1* and *MRR1* GOF mutations did not significantly affect *C. albicans* virulence compared to wild-type. However, *UPC2* GOF led to a significant decrease in virulence and reduced kidney fungal burden when compared to the wild-type strain (Lohberger, Coste and Sanglard 2014). Additionally, *UPC2* GOF mutations also delayed *C. albicans* filamentation upon phagocytosis by murine macrophages, which may partly explain the virulence defects associated with this mutation *in vivo* (Lohberger, Coste and Sanglard 2014). Interestingly, a strain combining *UPC2* GOF alleles with the GOF mutation in *MRR1* did not rescue the *UPC2* virulence defect, but rather attenuated virulence further (Lohberger, Coste and Sanglard 2014). Given

that azole resistance related to *UPC2/ERG11* overexpression is a common problem in the clinic it is possible that cells can compensate for the negative fitness effect of this GOF to thrive under host-imposed conditions (Flowers *et al.* 2012; Lohberger, Coste and Sanglard 2014).

FKS mutations at two 'hot spots' are a major fungal solution for generating echinocandin resistance. For example, *FKS2* T1987C enhances *C. glabrata* echinocandin resistance, but at the expense of *in vitro* fitness (Singh-Babak *et al.* 2012). Cells harboring this allele had a growth defect compared to wild-type in the absence of selection. However, this defect could be compensated by a GOF mutation to *CDC55* (C463T), which is one of the few characterized compensatory mutations for rescuing fitness in antifungal resistant isolates (Singh-Babak *et al.* 2012). *Saccharomyces cerevisiae* has been used as a model system to investigate acquired resistance via FKS mutations identified in echinocandin-resistant *Candida* and other fungal species (Johnson, Katiyar and Edlind 2011). This model has successfully replicated echinocandin resistance driven by mutations observed in *Candida parapsilosis* and *Fusarium solani* FKS genes. Whether this model could be used to help identify adjuvant compounds to improve echinocandin efficacy remains to be seen.

Candida glabrata has an additional mechanism for rapidly generating potentially advantageous mutations during drug treatment that involves altering the mismatch repair and double-strand break pathways. Mutations in *MSH2*, a gene involved in mismatch repair, were identified in ~55% of *C. glabrata* clinical isolates (Healey *et al.* 2016). These mutations conferred a hypermutable phenotype resulting in elevated resistance to azoles and echinocandins *in vitro*. *MSH2* deletion increased echinocandin resistance *in vivo*, though this *C. glabrata* strain was partially out-competed by wild-type in a mixed inocula murine gastrointestinal colonization model (Healey *et al.* 2016). Mutations in mismatch repair and double-strand DNA break repair genes in *C. albicans* also give rise to drug resistance more rapidly than wild-type cells (Legrand *et al.* 2007).

TRANSCRIPTOMICS, PROTEOMICS AND METABOLOMICS INSIGHTS INTO ANTIFUNGAL ADAPTATION

Is antifungal resistance a feature of phenotypic heterogeneity within populations, is it adaptation to specific drug insults or is it a combination of these processes? To address this question, 'omics studies have explored timed responses of drug-susceptible and drug-resistant populations to antifungals (Tables 2–4). While we found many studies that investigated adaptation using qRT-PCR and other targeted analyses, this section will discuss only 'omics-driven research into antifungal adaptation.

Azoles

Perhaps unsurprisingly, the majority of the antifungal 'omics studies that we identified for *S. cerevisiae*, *C. glabrata* and *C. albicans* characterized responses to azoles (i.e. fluconazole, clotrimazole, ketoconazole, miconazole, itraconazole and voriconazole). We submitted these datasets to the *Saccharomyces* Genome Database and *Candida* Genome Database GO Slim Mappers (Cherry *et al.* 2012; Skrzypek *et al.* 2017) to identify the top biological processes that were differentially regulated during drug treatment (Fig. 2A).

A total of two studies were of particular interest because they simultaneously analysed transcriptional responses for *S. cerevisiae* and *C. glabrata* to fluconazole or ketoconazole, respectively (Kuo *et al.*

Table 2. List of *S. cerevisiae* 'omics datasets with a brief description of methodology. A total of two studies include *C. glabrata* datasets. (Abbreviations: 5-FC, 5-fluorcytosine; FCZ, fluconazole; MCN, miconazole; CTZ, clotrimazole; KCZ, ketoconazole; ICZ, itraconazole; CSP, caspofungin and AmB, amphotericin B.)

Citation	Species	Analysis	Strain	Drug	Methods details
Zhang et al. (2002)	<i>S. cerevisiae</i>	Transcriptomics (Microarray)	L1190	5-FC, 25 µg/mL	OD ₆₀₀ ~0.8, 30°C YPD, exposed to 5-FC for 90 min, n = 1
Agarwal et al. (2003)	<i>S. cerevisiae</i>	Transcriptomics (Microarray)	S288c	AmB, 0.12 µg/mL	OD ₆₀₀ ~0.2, 30°C, SD, exposed to drug for 3 h, n = 2
Reinoso-Martin et al. (2003)	<i>S. cerevisiae</i>	Transcriptomics (Microarray)	BY4741	5-FC, 0.3 µg/mL CSP, 0.02 µg/mL KCZ, 56 µg/mL CSP, 10 ng/mL	OD ₆₀₀ ~1, 30°C YPD, exposed to CSP for 1, 2 and 3 h, n = 4
Kuo et al. (2010)	<i>S. cerevisiae</i> , <i>C. glabrata</i>	Transcriptomics (Microarray)	BY4741 CBS138	FCZ, 4 µg/mL (MIC ₅₀)	OD ₆₀₀ ~0.05–0.2, 30°C YPD, cells harvested 0, 1/3, 2/3, 1, 2 or 4 doubling times, n = 3
Nishikawa et al. (2016)	<i>S. cerevisiae</i> ,	Transcriptomics (RNA-Seq)	BY4741	KCZ, 40 µM	OD ₆₀₀ ~0.8, 30°C YPD, treated with DMSO 8 h, then KCZ for 15 min; n = 3
Pang et al. (2017)	<i>C. glabrata</i> <i>S. cerevisiae</i>	Transcriptomics (RNA-Seq)	DSY562 S288c	AmB, 0.03 µg/mL	30°C RPMI-1640, 50–60 min drug treatment; n = 3
Garcia et al. (2017)	<i>S. cerevisiae</i>	Transcriptomics (Microarray)	BY4741	CSP, 15 ng/mL	OD ₆₀₀ ~0.2, 30°C YPD, 2 h drug treatment; n = 3
ATripathi et al. (2020)	<i>S. cerevisiae</i>	Transcriptomics (RNA-Seq)	S288c	CSP, 0.03 µg/mL	OD ₆₀₀ ~0.1, 30°C SD +/- drug for ~4 h, n = 3
Messner et al. (2021)	<i>S. cerevisiae</i>	Proteomics (ScanningSWATH)	BY4741	MCN, KCZ, ICZ and CTZ, 10 µM	Overnight 30°C SD transferred to 96-well plate, exposed to drug overnight; n = 3–4

Table 4. List of *C. albicans* 'omics datasets with a brief description of methodology. (Abbreviations: FCZ, fluconazole; MCN, miconazole; KCZ, ketoconazole; CSP, caspofungin and AmB, amphotericin B.)

Citation	Species	Analysis	Strain	Drug	Methods details
Liu et al. (2005)	<i>C. albicans</i>	Transcriptomics (Microarray)	SC5314	KCZ, 19.13 µg/mL AmB, 0.029 µg/mL CSP, 0.0075 µg/mL 5-FC, 0.098 µg/mL	OD ₆₀₀ ~0.2, 30°C SD, 3 h, n = 3
Vasicek et al. (2014)	<i>C. albicans</i>	Transcriptomics (Microarray)	SC5314	FCZ, 10 µg/mL	OD ₆₀₀ ~0.05, 30°C YPD, 6 h, n = 2
Keller et al. (2015)	<i>C. albicans</i>	Transcriptomics (Microarray)	SC5314	FCZ, ~0.5 µg/mL (IC ₅₀)	OD ₆₀₀ ~0.4, 30°C RPMI + 10% fetal calf serum, 3 h, n = 3
de Cremer et al. (2016)	<i>C. albicans</i>	Transcriptomics (RNA-Seq)	SC5314	MCN, 75 µM	Pre-formed biofilms +/- drug at 37°C in RPMI, 4 + 24 h, n = 3
Shivarathri et al. (2019)	<i>C. albicans</i>	Transcriptomics (RNA-Seq)	SC5314	CSP, 10 ng/mL	30°C YPD, 15 + 45 min, n = 3
Kuloyo et al. (2020)	<i>C. albicans</i>	Transcriptomics (RNA-Seq)	SC5314	FCZ, 1 µg/mL	RPMI 37°C, adhered to polystyrene 90 min, +/- drug 6 h, n = 3
Hoehamer et al. (2010)	<i>C. albicans</i>	Proteomics (MALDI-ToF)	SC5314	KCZ, 19.13 µg/mL	OD ₆₀₀ ~0.2, 30°C SD, 6 h, n = 3
Sorgo et al. (2011)	<i>C. albicans</i>	Proteomics (LC-ESI-MS/MS)	SC5314	AmB, 0.029 µg/mL CSP, 0.0075 µg/mL FCZ, 0.5 µg/mL	OD ₆₀₀ ~0.05, 37°C YNB-S, 18 h, n = 5
Ene et al. (2012)	<i>C. albicans</i>	Proteomics (LC-MS/MS)	RM1000	Ambisome, 10 µg/mL	OD ₆₀₀ ~0.1, YNB + 2% glucose or lactate, 1 h, n = 3
Katragkou et al. (2016)	<i>C. albicans</i>	Metabolomics (GC-MS, UHPLC-Q-TOF/MS and HILIC-QQQ/MS)	SC5314	CSP, 0.08 µg/mL MCN, 25 µg/mL KCZ, 16 µg/mL	FCZ sensitive and resistant (64 µg/mL) strains, 30°C YPD to 10 ⁸ cells/mL, n = 6

Table 3. List of *C. glabrata* antifungal 'omics datasets with a brief description of methodology. (Abbreviations: 5-FC, 5-fluocytosine; FCZ, fluconazole; CTZ, clotrimazole; KCZ, ketoconazole and AmB, amphotericin B.)

Citation	Species	Analysis	Strain	Drug	Methods details
Caudle (unpublished data)	<i>C. glabrata</i>	Transcriptomics (Microarray)	200989	FCZ, 64 µg/mL	OD ₆₀₀ ~0.2, 30°C YPD, +/- drug 2.5 h, n = 3
Kuo et al. (2010)	<i>S. cerevisiae</i> , <i>C. glabrata</i>	Transcriptomics (Microarray)	BY4741 CBS138	FCZ, 4 µg/mL MIC ₅₀	OD ₆₀₀ ~0.05–0.2, 30°C YPD, cells Harvested 0, 1/3, 2/3, 1, 2 or 4 doubling times, n = 3 (2x technical)
Nishikawa et al. (2016)	<i>S. cerevisiae</i> , <i>C. glabrata</i>	Transcriptomics (RNA-Seq)	BY4741 DSY562	KCZ, 40 µM	OD ₆₀₀ ~0.8, 30°C YPD, treated with DMSO 8 h, then KCZ for 15 min; n = 3
Pais et al. (2020)	<i>C. glabrata</i>	Transcriptomics (RNA-Seq)	KUE100	FCZ, 150 µg/mL	30°C basal medium to mid-exponential phase, treated +/- drug 1 h, n = 3 (2x technical)
Alves et al. (2020)	<i>C. glabrata</i>	Transcriptomics (RNA-Seq)	CBS138	FCZ, 1250 µg/mL	Pre-formed biofilms +/- drug at 37°C in RPMI pH 7.0, 24 h, n = 3
Pais et al. (2016a)	<i>C. glabrata</i>	Proteomics (iTRAQ-MS)	66032	5-FC, 4 µg/mL	30°C Basal medium, +/- 5-FC 1 h, n = 3
Pais et al. (2016b)	<i>C. glabrata</i>	Proteomics (iTRAQ-MS)	66032	CTZ, 100 µg/mL	OD ₆₀₀ ~0.4, 30°C basal medium, 1 h, n = 3

al. 2010; Nishikawa et al. 2016). Both fluconazole and ketoconazole induced significant changes in gene expression associated with lipid and carbohydrate metabolism, induction of transmembrane transporters such as drug transporters and down-regulation of genes involved in rRNA processing or ribosome biogenesis (Kuo et al. 2010; Nishikawa et al. 2016). These categorical changes in gene expression were also common features in other azole datasets for *C. glabrata* (Caudle 2010; Pais et al. 2020), *C. albicans* (Liu et al. 2005; Vasicek et al. 2014; Weil et al. 2017) and for both *C. glabrata* and *C. albicans* grown under biofilm-forming conditions (Alves et al. 2020; Kuloyo et al. 2020). All three species down-regulated gene expression associated with DNA replication during fluconazole treatment in multiple datasets (Kuo et al. 2010; Alves et al. 2020; Kuloyo et al. 2020), which correlates well with *in vitro* data demonstrating slower growth rates during drug-induced stress (Rosenberg et al. 2018). These changes in gene expression largely match our expectations for adaptation to azoles, which would involve alterations in lipid metabolism to remedy the lack of membrane sterols or build-up of toxic intermediates and an attempt to increase membrane transporters to eliminate antifungals from the cytoplasm.

The consistency between these studies is even more remarkable because of the different approaches used: Caudle's study used a clinical isolate of *C. glabrata*, Weil et al. (2017) investigated *C. albicans* strains with mistranslation mutations that affected azole resistance and most studies used different concentrations of drug, growth media or time points for analysis. However, looking more globally at the differentially expressed gene datasets, there were some key differences between studies. For example, Kuloyo et al. (2020) observed that *C. albicans* biofilms treated with fluconazole down-regulated genes involved in filamentous growth, but Vasicek et al. (2014) and Liu et al. (2005) observed induction of filamentous growth genes for fluconazole or ketoconazole-treated planktonic cells, respectively. *Candida glabrata* heme and iron homeostasis were altered in fluconazole-treated cells (Caudle 2010; Pais et al. 2020), but these processes did not appear to be significantly impacted in the GO Slim analysis for *S. cerevisiae* and *C. albicans*.

Other 'omics studies have corroborated key aspects of available transcript profiling data. For example, the mevalonate pathway

provides important precursors for ergosterol biosynthesis. Consistent with changes in lipid metabolism, *C. albicans* metabolomics data during fluconazole treatment shows a build-up in mevalonate pathway by-products due to the block in ergosterol synthesis (Katragkou et al. 2016). The metabolomics data also indicate that *C. albicans* undergoes major changes in central carbon metabolism and decreases amino acid metabolism, though the significance of these changes is unclear. Proteomics studies have characterized the basal prevalence of cytoplasmic and membrane proteins in azole-resistant and azole-susceptible isolates under the working hypothesis that drug-resistant strains will have enriched expression of drug resistance markers, such as efflux pumps. Consistent with the data obtained from drug stress imposed on sensitive cells, *C. albicans* strains that are resistant to fluconazole had enriched expression of proteins associated with lipid metabolic processes (Hooshdaran et al. 2004) and decreased prevalence of proteins involved in DNA repair. *Candida glabrata* fluconazole-resistant isolates were enriched for proteins involved in drug efflux and metabolic processes (Shen et al. 2015). Unfortunately, a handful of studies on *C. glabrata* azole-resistant isolates are missing specific gene identifying information, but similarly indicated by biological process data that proteins involved in glucose metabolism and cell wall biogenesis were differentially expressed in azole-resistant strains compared to azole-sensitive cells (Loureiro Y Penha et al. 2010; Yoo et al. 2012, 2013).

Proteomics work with drug-sensitive strains has focused largely on determining membrane or cell wall changes in protein levels in response to azoles. Membrane proteomics have corroborated transcriptional studies on *C. glabrata* adaptation to azoles. For example, clotrimazole treatment induced drug transporter expression, including Tpo1, Ssq2 and Pdr5, and downregulated expression of proteins associated with ribosome biogenesis and oxidative phosphorylation pathways (Pais et al. 2016b). *Candida albicans* cell wall proteomics studies demonstrated that fluconazole, miconazole and ketoconazole differentially regulated the expression of several cell wall proteins and virulence factors including adhesins (ALS3 and ALS4), GPI-anchored proteins (PGA4 and PGA31) and secreted aspartyl proteases (SAP7 and SAP9; Sorgo

et al. 2011; Ene et al. 2012). Proteomic data on *C. glabrata* virulence factor expression, such as EPA adhesins, in response to azole treatment is lacking. However, transcriptomic data suggests that CgEPA1, a sub-telomerically encoded adhesin that plays an important role in human epithelial cell adhesion, is upregulated in multiple fluconazole-resistant clinical isolates compared to fluconazole-sensitive isolates (Caudle 2010). CgEPA1 is a homolog of ScFLO10, a flocculin important for cell-to-cell adhesion. ScFLO10 also is upregulated in yeast cells grown in the presence of fluconazole (Kuo et al. 2010). Cell-to-cell and cell-to-substrate adhesion are important for biofilm formation, which can modulate antifungal efficacy. It remains to be seen whether azole-induced adhesin expression constitutes a concerted effort to form biofilms as part of a protective adaptive response to azole exposure.

Echinocandins

Echinocandins are the preferred first line of treatment for invasive candidiasis. However, out of the six studies that used 'omics techniques to interrogate echinocandin adaptation in fungal cells, none included *C. glabrata*. Further, all of the studies we found focused on characterizing responses to caspofungin but not anidulafungin or micafungin.

In 2003, back-to-back microarray studies in *S. cerevisiae* provided a first glimpse of yeast adaptive responses to caspofungin (Agarwal et al. 2003; Reinoso-Martin et al. 2003). Unsurprisingly, genes involved in cell wall organization or biogenesis were the most significantly enriched biological process during caspofungin treatment (Agarwal et al. 2003; Reinoso-Martin et al. 2003). Caspofungin also induced the expression of genes involved in sporulation or 'response to chemical' in both datasets (Agarwal et al. 2003; Reinoso-Martin et al. 2003). In contrast, genes involved in transmembrane or ion transport were down-regulated in response to caspofungin treatment. *In vitro* data, thus far supports the conclusion that echinocandins are not substrates for the ABC transporters that mediate azole efflux in azole-resistant strains (Niimi et al. 2006). More recent transcript profiling studies (Garcia et al. 2017; Tripathi et al. 2020) show consistent changes in gene expression with those described by Agarwal et al. (2003). In particular, genes involved in cell wall biogenesis, carbohydrate metabolism and protein phosphorylation/modification were enriched during caspofungin treatment while genes involved in conjugation and ion or transmembrane transport were down-regulated (Agarwal et al. 2003; Garcia et al. 2017; Tripathi et al. 2020). Reinoso-Martin et al. (2003) showed enrichment of cell cycle and DNA replication machinery while Agarwal et al. (2003) observed enrichment in genes involved in carbohydrate and amino acid metabolism.

Proteomics data from *C. albicans* during caspofungin treatment presents some similarities to the *S. cerevisiae* datasets. In *C. albicans*, proteins involved in carbohydrate metabolism, response to chemical and cell-cycle regulation were enriched during caspofungin exposure in two studies (Liu et al. 2005; Hoehamer et al. 2010). Shivarathri et al. (2019) investigated *C. albicans* responses to caspofungin over 15 and 45 min of exposure. Unique to *C. albicans*, caspofungin treatment differentially regulated filamentous growth gene expression with key genes involved in hyphal growth (HGC1, RFX2 and UME6) up-regulated within 45 min of drug exposure (Shivarathri et al. 2019). Similar to *S. cerevisiae*, *C. albicans*-induced expression of genes involved in carbohydrate metabolism and response to stress and down-regulated the expression of genes associated with lipid metabolism, protein catabolism and cellular homeostasis (Shivarathri et al. 2019).

In vitro and *in vivo* data from *C. albicans* paints a striking image of how caspofungin affects cell viability and virulence. Cells starved for cell wall β -glucan due to inhibited synthesis compensate by dramatically increasing chitin content in the inner cell wall (Lee et al. 2012; Walker, Gow and Munro 2013). This alteration in inner cell wall composition has consequences for innate immune interactions and virulence. *Candida albicans* cells treated with caspofungin are hypovirulent in mice, but do not appear to be cleared by immune cells and replicate to high fungal burdens in murine kidneys (Lee et al. 2012). Interestingly, the compensatory adaptation in chitin synthesis in response to caspofungin is not conserved in *C. glabrata*, though cell wall integrity appears to be important for *in vivo* echinocandin tolerance (Garcia-Rubio et al. 2021). In *S. cerevisiae*, a deletion library screen identified 25 mutations that resulted in enhanced caspofungin resistance (Garcia et al. 2015). Mutations related to lipid metabolism (CSG2, ELO2, ELO3, CKA2 and SUR1), sterol biosynthesis (SAY1, ERG3 and NSG2), fatty acid synthesis (ETR1), translocation of phospholipids across the plasma membrane (LEM3) and lower glucan synthase activity (WSC1, ELO2 and ELO3) conferred hyper-resistance to caspofungin (Garcia et al. 2015). Understanding physical and genetic adaptation mechanisms to echinocandins and their conservation across species could provide useful insights into how to overcome resistance through adjuvant therapy targeted against key adaptive traits, such as other cell wall biogenesis pathways.

Polyenes

AmB is an effective and robust last line of defense against invasive fungal infections. Given its length of use in the clinic, we were surprised to find few 'omics studies on AmB responses and adaptation in *S. cerevisiae* or *Candida* species.

A total of two studies, ~14-years-apart, investigated AmB effects on *S. cerevisiae* transcription using microarray (Agarwal et al. 2003) and RNA-Seq (Pang et al. 2017) approaches. Both studies have notable consistency in the biological processes enriched by treatment with AmB, which included genes involved in transmembrane and ion transport, cell wall organization, amino acid metabolism and transcription by RNA polymerase II (Agarwal et al. 2003; Pang et al. 2017). Both studies also noted down-regulation of genes involved in cell-cycle progression. Agarwal et al. (2003) observed differential regulation of carbohydrate metabolism and cytoskeletal organization genes while Pang et al. (2017) reported changes in gene expression related to lipid metabolic processes, mitochondrion organization, rRNA and tRNA processing and ribosome biogenesis.

In *C. albicans*, AmB adaptation has been investigated using proteomics to determine changes in cell wall and cytoplasmic protein levels (Hoehamer et al. 2010; Ene et al. 2012). Hoehamer et al. (2010) identified several proteins that were enriched during AmB treatment that are consistent with transcriptional changes noted by Agarwal et al. (2003) in *S. cerevisiae*. In particular, proteins involved in nucleobase, carbohydrate and amino acid metabolism, transmembrane transport and response to oxidative stress were more prevalent in *C. albicans* cells exposed to AmB compared to untreated cells. Ene et al. (2012) characterized changes in cell wall protein expression during AmB treatment and discovered that proteins involved in β -glucan maintenance (Phr2, Crh11 and Eng1) were enriched during drug exposure. Other cell wall proteins were less prevalent during drug exposure, including the chitinase Cht1, secreted aspartyl protease Sap9 and virulence factor Rbt4 (Ene et al. 2012).

Polyene perturbations to membrane fluidity and homeostasis clearly have large effects on lipid metabolism, membrane protein incorporation and cell wall organization. Ene *et al.* (2012) also highlights how AmB treatment may have the added benefit of negatively regulating virulence factor expression. While clinical resistance to AmB is rare, further study on the adaptation of fungal pathogens to this drug is warranted especially as the first observed cases of pan-resistant *C. auris* are being reported in the USA (Lyman *et al.* 2021).

5-FC

Finally, we identified two *S. cerevisiae* microarray studies, one *C. albicans* microarray study and one *C. glabrata* proteomics study that investigated cell responses to treatment with 5-FC.

Saccharomyces cerevisiae microarray investigations in 2002 and 2003 showed that 5-FC treated cells responded to drug insult by up-regulating gene expression associated with DNA replication, DNA repair and cell-cycle machinery (Zhang *et al.* 2002; Agarwal *et al.* 2003). Transmembrane and ion transporters were differentially regulated. Some transport classes, such as Mep ammonium transporters, were down-regulated during drug treatment and other genes, such as antiporter family member TPO2, were up-regulated (Agarwal *et al.* 2003). Genes involved in amino acid metabolism and transcription via RNA polymerase II were down-regulated during 5-FC exposure (Zhang *et al.* 2002; Agarwal *et al.* 2003).

The transcript profiling data from *C. albicans* and proteomics data from *C. glabrata* cells treated with 5-FC bear little resemblance to *S. cerevisiae* transcript profiling (Liu *et al.* 2005; Pais *et al.* 2016a). Similar to *S. cerevisiae*, transmembrane transporters were differentially regulated by 5-FC treatment, but the most enriched biological processes in *C. albicans* and *C. glabrata* involved translational regulation and ribosome biogenesis rather than DNA repair. In fungal cells, 5-FC is converted to 5-FU, which is further converted into several metabolites that affect translation and cause DNA damage. What these datasets appear to suggest is that 5-FC treatment differentially affects *Candida* species and *S. cerevisiae* biological responses (Zhang *et al.* 2002; Agarwal *et al.* 2003; Liu *et al.* 2005; Pais *et al.* 2016a). *Candida glabrata* and *C. albicans* appear to be more sensitive to translational inhibition caused via 5-FU incorporation into mRNA, whereas *S. cerevisiae* transcriptional changes indicate sensitivity to the depletion of dTTP via 5-FU inhibition of thymidylate synthase, resulting in dUTP incorporation into DNA and, ultimately, DNA damage. Further transcriptomics and proteomics work are needed to confirm these observations of differing sensitivities to 5-FC in *C. albicans*, *C. glabrata* and *S. cerevisiae*.

CONCLUSIONS

In this review, we have discussed the resistance mechanisms and 'omics-determined physiological responses of *S. cerevisiae*, *C. glabrata* and *C. albicans* to the major classes of antifungal drugs used against invasive candidiasis. Some resistance mechanisms and adaptive responses are conserved between these pathogenic and non-pathogenic fungi, particularly against azole treatment, where cells showed adaptation in lipid metabolism and enrichment of efflux pump expression (Fig. 2). Some adaptive mechanisms were less well-conserved, such as the datasets suggesting that *C. albicans* and *C. glabrata* responses to 5-FC were driven more by translational inhibition compared to *S. cerevisiae*, which appeared to preferentially up-regulate genes involved in DNA repair (Fig. 2B and supplemental data). These observations indicate that

S. cerevisiae may be an excellent model organism for understanding responses to certain antifungals but may be more difficult to extrapolate data for others. Additional investigations on model and non-model organism antifungal responses are needed to address the limitations of basing antifungal response paradigms on data from normally non-pathogenic organisms.

We initially set out to perform a systematic review of 'omics datasets on antifungal adaptation. However, our efforts to conduct this review systematically were hindered by three fundamental issues. First, sourcing articles using broad keyword search strings, such as the use of 'antifungal' AND 'transcriptomics' AND 'species', returned fewer than 10% of the relevant articles highlighted in this review. Substituting 'antifungal' with a specific antifungal name only modestly improved search success. Second, while there is an abundance of 'omics literature on azole adaptation, differences in strains, media, growth conditions and timepoints used made data comparisons difficult. We have attempted to address this issue by doing light-touch comparisons of differentially regulated biological processes from each study and highlighting consistencies between studies which we consider even more robust given the technical differences in approaches. Finally, compared to the azole literature there is a relative drought of information for micafungin, anidulafungin, isavuconazole, voriconazole, AmB and 5-FC. What the field needs in the future are large-scale studies covering multiple timepoints, strains and antifungal drugs to help draw more robust conclusions about how antifungals influence fungal adaptation, host interactions and the development of antifungal resistance. Next-generation technologies, such as single-cell RNA sequencing and multi-omics approaches, will be important tools to address the dynamic sub-population changes behind the development of tolerance vs. mutational approaches to surviving antifungals.

Antifungals significantly affect several aspects of fungal physiology including carbon and lipid metabolism, cell wall organization, membrane protein expression, cell division and genomic stability. Each of these processes in turn can affect fungal fitness, host interactions and pathogenesis. As we have discussed earlier in this review, there are several mechanisms that lead to resistance and survival in the face of antifungal insults, though the mechanisms driving tolerance are poorly understood. We are approaching a clinical cliff where the limited repertoire of available antifungals is coming up short against emerging pan-resistant fungal pathogens. We need carefully designed 'omics and multi-omics studies to better understand how genetic and physiological rewiring events during drug exposure alter antifungal resistance and host interactions to identify new avenues for the development of adjuvant or novel therapeutic strategies.

SUPPLEMENTARY DATA

Supplementary data are available at [FEMSYR](https://www.femsyr.com) online.

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