REVIEW

Stress adaptation in a pathogenic fungus

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ABSTRACT

Candida albicans is a major fungal pathogen of humans. This yeast is carried by many individuals as a harmless commensal, but when immune defences are perturbed it causes mucosal infections (thrush). Additionally, when the immune system becomes severely compromised, C. albicans often causes life-threatening systemic infections. A battery of virulence factors and fitness attributes promote the pathogenicity of C. albicans. Fitness attributes include robust responses to local environmental stresses, the inactivation of which attenuates virulence. Stress signalling pathways in C. albicans include evolutionarily conserved modules. However, there has been rewiring of some stress regulatory circuitry such that the roles of a number of regulators in C. albicans have diverged relative to the benign model yeasts Saccharomyces cerevisiae and Schizosaccharomyces pombe. This reflects the specific evolution of C. albicans as an opportunistic pathogen obligately associated with warm-blooded animals, compared with other yeasts that are found across diverse environmental niches. Our understanding of C. albicans stress signalling is based primarily on the in vitro responses of glucose-grown cells to individual stresses. However, in vivo this pathogen occupies complex and dynamic host niches characterised by alternative carbon sources and simultaneous exposure to combinations of stresses (rather than individual stresses). It has become apparent that changes in carbon source strongly influence stress resistance, and that some combinatorial stresses exert non-additive effects upon C. albicans. These effects, which are relevant to fungus-host interactions during disease progression, are mediated by multiple mechanisms that include signalling and chemical crosstalk, stress pathway interference and a biological transistor.

KEY WORDS: Candida albicans, Fungal pathogenicity, Heat shock, Oxidative stress, Nitrosative stress, Osmotic stress, Cationic stress, Stress adaptation, Carbon metabolism

Introduction: Candida albicans – an opportunistic pathogen of humans

Candida albicans is a major fungal pathogen of humans that occupies a wide range of divergent niches within the host. It normally exists as a harmless commensal organism in the microflora of the skin, oral cavity, and gastrointestinal and urogenital tracts of most healthy individuals (Odds, 1988; Calderone, 2002; Calderone and Clancy, 2012). However, C. albicans frequently causes oral and vaginal infections (thrush) when the microflora is disturbed by antibiotic usage or when immune defences are perturbed, for example in HIV patients (Sobel, 2007; Revankar and Sobel, 2012). In individuals whose immune systems are severely compromised (such as neutropenic patients undergoing chemotherapy or transplant surgery), the fungus can survive in the bloodstream, leading to the colonisation of internal organs such as the kidney, liver, spleen and brain (Pfaller and Diekema, 2007; Calderone and Clancy, 2012). Candida is the fourth most common cause of hospital-acquired bloodstream infections, over half of which can be fatal in some patient groups (Perlroth et al., 2007). This high morbidity exists despite the availability of specialised antifungal drugs such as the azoles, polyenes and echinocandins (Odds et al., 2003a; Brown et al., 2012b), reflecting the challenges in diagnosing systemic fungal infections, the resultant delays in treatment, and the limited choice of effective antifungal drugs (Pfaller and Diekema, 2010; Brown et al., 2012b). From the fungal perspective, it is clear that C. albicans can adapt effectively to diverse host niches.

The evolutionary history of C. albicans has established both its pathogenic behaviour and also its properties as an experimental system. Candida albicans is a member of the ascomycete phylum, which includes the model yeasts Saccharomyces cerevisiae and Schizosaccharomyces pombe. These benign model yeasts provide paradigms against which C. albicans is often compared (Berman and Sudbery, 2002; Enjalbert et al., 2006; Noble and Johnson, 2007). However, in evolutionary terms C. albicans is only distantly related to S. cerevisiae (circa 150 million years) and S. pombe (circa 400 million years) (Galagan et al., 2005), and the latter evolutionary distance represents greater separation than exists between humans and sharks. Furthermore, although ascomycetes are generally defined by their packaging of sexual spores into an ascus structure, C. albicans has not been observed to undergo meiosis to generate spores. Rather, this diploid yeast, which until very recently was thought to be constitutively diploid (Hickman et al., 2013), displays a complex parasexual cycle. Candida albicans must undergo homoyogosity at the mating type locus (MTL) and then undergo an episomatic switch to mating competent cells (the opaque form) before it matures to form tetraploids (Noble and Johnson, 2007). This is followed by chromosome loss to return to the diploid state (Forche et al., 2008). While parasexual recombination could have contributed to the recent evolution of C. albicans, the population structure is predominantly clonal (Cowan et al., 2002; Odds et al., 2007). Indeed, its recent evolution appears to have been driven largely by its clonal behaviour as a pathogen. Candida albicans has not been associated with any particular environmental niche and hence is thought to be obligately associated with warm-blooded animals (Odds, 1988). Therefore, it is not surprising that this fungus
has undergone the rapid evolution of virulence factors and fitness attributes associated with its pathogenicity (Butler et al., 2009; Nikolaou et al., 2009) as well as evolutionary rewiring of transcriptional and post-transcriptional circuitries relative to S. cerevisiae (Ihmels et al., 2005; Martchenko et al., 2007; Lavoie et al., 2009; Baker et al., 2012; Sandai et al., 2012). These changes have had a significant impact on the evolution of stress adaptation in C. albicans (Brown et al., 2012a).

The loss of a bona fide sexual cycle has had a major impact on the experimental dissection of C. albicans pathobiology. Researchers have had to rely mainly on genomic and molecular approaches, rather than genetic strategies to examine the virulence of this fungus (Noble and Johnson, 2007). Nevertheless, these approaches have revealed an armoury of virulence factors that promote the pathogenicity of C. albicans. Virulence factors have been defined as those fungal factors that interact directly with host components (Odds et al., 2003b). For example, reversible morphogenetic transitions between yeast, pseudohyphal and hyphal growth forms contribute to the virulence of C. albicans (Lo et al., 1997; Saville et al., 2003). Yeast forms are thought to promote dissemination, whereas the filamentous forms are better suited to penetrate tissue. Hyphae also display thigmotrophic responses that appear to contribute to tissue penetration (Sherwood et al., 1992; Brand, 2012). Initial colonisation is mediated by families of cell surface adhesins that promote adherence to host tissues (Staab et al., 1999; Hoyer et al., 2008). One of these adhesins, Als3, also acts as an invasin by promoting the invasion of endothelial cells (Phan et al., 2007), contributing to the assimilation of the essential micronutrient iron (Almeida et al., 2008; Almeida et al., 2009) and to brain colonisation (Liu et al., 2011). Candida albicans expresses additional factors involved in iron and zinc assimilation, some of which are essential for virulence (Almeida et al., 2009; Citiulo et al., 2012), and which are induced during renal infection (J.P. and A.J.P.B., unpublished). Candida albicans also secretes families of hydrolytic enzymes including proteases, lipases and phospholipases (Naglik et al., 2003; Schaller et al., 2005) that enhance tissue invasion, provide nutrients to support fungal growth and modulate host immune responses (Pietrella et al., 2010). These and other factors are temporally and spatially regulated during colonisation and disease progression, thereby enhancing C. albicans pathogenicity.

Additional factors promote the virulence of C. albicans without interacting directly with the host. These factors, which have been termed ‘fitness attributes’ (Brown, 2005), include functions involved in metabolic and stress adaptation and act by tuning the physiological fitness of C. albicans cells to their local host microenvironment. Two main types of evidence have highlighted the importance of stress adaptation for the virulence of C. albicans. First, numerous genome-wide expression profiles have demonstrated that stress genes are induced when the fungus comes in contact with the host. For example, oxidative, nitrosative and heat shock functions are induced when cells are phagocytosed by macrophages or neutrophils (Rubin-Bejerano et al., 2003; Lorenz et al., 2004; Fradin et al., 2005), and the niche-specific induction of specific stress responses has been confirmed by single cell profiling using diagnostic green fluorescent protein (GFP) fusions (Enjalbert et al., 2007; Miramón et al., 2012). Second, the virulence of C. albicans in mouse models of infection is attenuated by the inactivation of key stress functions such as the stress-activated protein kinase (SAPK) Hog1, the catalase Cat1 or the superoxide dismutase Sod1 (Wysong et al., 1998; Alonso-Monge et al., 1999; Hwang et al., 2002; Cheetham et al., 2011). Significant progress has been made in the elaboration of stress-adaptive responses, their regulation in C. albicans and their divergence from the corresponding pathways in model yeasts. A brief overview of these mechanisms will be discussed here. This provides the platform for the main theme of this review – stress adaptation in the context of complex and dynamic host niches (mentioned above), in which C. albicans cells must respond to multiple environmental inputs, rather than to the individual stresses commonly studied in vitro.

**Overview of stress adaptation mechanisms in C. albicans**

Stress signalling pathways are relatively well characterised in S. cerevisiae and S. pombe. A number of the key regulators are evolutionarily conserved in C. albicans (Butler et al., 2009; Nikolaou et al., 2009) (Fig. 1). However, the roles of some of these regulators have diverged (Enjalbert et al., 2003; Nicholls et al., 2004; Ramsdale et al., 2008; Cheetham et al., 2007), and C. albicans is relatively resistant to physiologically relevant stresses compared with model yeasts (Jamieson et al., 1996; Nikolaou et al., 2009). This is consistent with the idea that stress responses in C. albicans have been evolutionarily tuned to host niches. Stress signalling in C. albicans has been described in a number of recent reviews (Chauhan et al., 2006; Alonso-Monge et al., 2009b; Brown et al., 2009; Smith et al., 2010; Brown et al., 2012a). Therefore, the purpose of this section is to summarise key stress signalling pathways, highlighting their relevance to infection.

**Heat shock**

The heat shock response is ubiquitous in nature. In eukaryotes, it involves the induction of a defined set of heat shock proteins (HSPs), many of which promote the folding of client proteins or target aggregated or damaged proteins for degradation (Parsell and Lindquist, 1993; Feder and Hofmann, 1999). The response in C. albicans, as in other yeasts, is driven by the heat shock transcription factor Hsf1 (Nicholls et al., 2009). Hsf1 is conserved from yeasts to humans and is essential for viability (Sorger and Pelham, 1988; Sarge et al., 1993; Wu, 1995). In response to acute heat shock, C. albicans Hsf1 becomes phosphorylated and induces the expression of target heat shock protein (HSP) genes via canonical heat shock elements (HSEs) in their promoters (Nicholls et al., 2009), an interaction that is conserved in other eukaryotes (Sorger and Pelham, 1988; Jakobsen and Pelham, 1988; Holmberg et al., 2001). HSP gene induction leads to the refolding or degradation of damaged proteins, thereby promoting cellular adaptation to the thermal insult. Indeed, in C. albicans heat shock induces polyubiquitin (UBI4) expression, which is required for resistance to thermal stress (Roig and Gozalbo, 2003; Leach et al., 2011). The HSP90 gene is also activated in an Hsf1-dependent fashion (Nicholls et al., 2009). Heat shock protein 90 (Hsp90) has been described as a molecular transistor as it modulates the activity of client regulatory proteins (Leach et al., 2012a). Following thermal adaptation, Hsp90 interacts...
physically with Hsf1 to downregulate the heat shock response in *C. albicans* (Leach et al., 2012b) (Fig. 1).

Significantly, while other conserved stress regulatory circuits have undergone evolutionary rewiring (see below), heat shock regulation has been maintained in *C. albicans* (Nicholls et al., 2009) despite its obligate association with warm-blooded animals (Odds, 1988). Presumably the fungus occupies thermally buffered niches in the host and is generally sheltered from the acute heat shocks that are imposed in the laboratory. Interestingly, mutations that block the activation of the heat shock response attenuate the virulence of *C. albicans* (Nicholls et al., 2011). Mathematical modelling of the dynamic regulation of Hsf1 during thermal adaptation has provided an answer to this conundrum (Leach et al., 2012c). The Hsf1–HSE regulon appears to be activated even during slow thermal transitions such as those suffered by febrile patients. This explains why Hsf1 activation is essential for the virulence of *C. albicans* (Nicholls et al., 2011). Clearly, the Hsf1–HSE regulon is critical for the maintenance of thermal homeostasis, not merely for adaptation to acute heat shocks.

### Osmotic and cationic stress

Exposure to NaCl or KCl imposes osmotic and cationic stress, which causes rapid water loss, a reduction in cell size and loss of turgor pressure (Kühn and Klipp, 2012). This triggers the phosphorylation and nuclear accumulation of the SAPK Hog1, which in turn mediates the activation of target genes including those encoding glycerol biosynthetic enzymes (Smith et al., 2004; Enjalbert et al., 2006). This leads to the accumulation of glycerol, the restoration of turgor pressure and the resumption of growth. Glycerol biosynthetic gene induction, glycerol accumulation and the successful adaptation of *C. albicans* cells to osmotic/cationic stresses are Hog1 dependent (San José et al., 1996; Smith et al., 2004).

Hog1 is a component of a highly conserved mitogen-activated protein (MAP) kinase pathway involved in osmo-adaptation in other yeasts (Nikolaou et al., 2009; Smith et al., 2010). In *C. albicans*, this MAP kinase (MAPK) is activated by the MAP kinase kinase (MAPKK) Pbs2, which in turn is activated by a single MAP kinase kinase kinase (MAPKKK), Ssk2 (Arana et al., 2005; Cheetham et al., 2007) (Fig. 1). However, the upstream regulators that activate this MAPK module in response to osmotic stress have not been established unambiguously in *C. albicans*. In *S. cerevisiae*, this MAPK module responds to two well-defined upstream branches (reviewed by Smith et al., 2010). The Sho1 branch activates Hog1 signalling via Cdc42, Ste50, Ste20 and Cla4, and through the MAPKKK Ste11 specifically in response to heat or osmotic stress. The Sn1l phospho-relay system includes Ypd1 and Ssk1, and activates Hog1 signalling via the MAPKKKS Ssk2 and Ssk22 in response to a broad range of stresses, including osmotic stress. *Candida albicans* has orthologues for many of these proteins (Nikolaou et al., 2009), as well as proteins that are related to histidine kinases in *S. cerevisiae* and *S. pombe* (*C. albicans* Sln1, Chk1, Nik1) (Krupa and Calderone, 2006). However, in *C. albicans* none of these histidine kinases or Ssk1 is essential for the osmotic stress-induced activation of Hog1 (Chauhan et al., 2003; Krupa and Calderone, 2006), suggesting that the Sln1 branch does not transduce osmotic stress signals to Hog1. Furthermore, a *ypd1 sho1* double mutation does not block osmotic stress signalling to Hog1 in *C. albicans* (Román et al., 2005), indicating that the Sho1 branch is not essential for osmotic stress signalling either. Therefore, it is not yet clear how osmotic stress signals are transduced to Hog1, and there appears to have been significant evolutionary rewiring of the upstream regulators of this stress pathway.

The inactivation of Hog1 attenuates the virulence of *C. albicans* (Alonso-Monge et al., 1999; Cheetham et al., 2011). However, this is not attributable simply to the loss of osmotic or cationic stress adaptation because Hog1 has been shown to execute additional functions. Hog1 is required for adaptation to other stresses, modulates cellular morphogenesis, influences metabolism and affects cell wall functionality (Alonso-Monge et al., 1999; Alonso-Monge et al., 2003; Alonso-Monge et al., 2009a; Smith et al., 2004; Eisman et al., 2006). Nevertheless, several observations suggest that osmotic and cationic stress adaptation play significant roles in certain host niches. First, NaCl concentrations can approach 600 mmol l⁻¹ in the kidney and be high in the urine (Ohno et al., 1997; Zhang et al., 2004). Second, *C. albicans* cells are exposed to K⁺ fluxes following phagocytosis by host immune cells (Da Silva-Santos et al., 2002; Fang, 2004). Third, mathematical modelling of osmotic stress adaptation in *S. cerevisiae* has highlighted the role of this regulatory apparatus in mediating cellular osmo-homeostasis and the maintenance of water balance (Klipp et al., 2005), in addition to its role in adaptation to the acute osmotic shocks that experimentalists tend to impose *in vitro*. Hence, Hog1-mediated osmotic adaptation is likely to be required in many host niches.

### Cell wall stress

Antifungal drugs such as caspofungin and chemicals such as Calcofluor White and Congo Red are often used to exert stress upon the cell wall of *C. albicans in vitro* (Wiederhold et al., 2005; Eisman
et al., 2006; Walker et al., 2008; Leach et al., 2012a). Caspofungin and Congo Red interfere with β-glucan synthesis and assembly, whereas Calcofluor White perturbs chitin assembly. The cell wall changes that occur in response to these artificial insults presumably reflect normal cell wall homeostasis during growth and development in the wild, as well as cell wall remodelling events that occur in response to stresses encountered during host–fungus interactions. The Hog1 pathway contributes to cell wall functionality and regulates chitin biosynthetic functions (Eisman et al., 2006; Munro et al., 2007). Two additional MAPK pathways contribute to cell wall stress resistance in C. albicans: the cell integrity pathway (defined by the MAPK Mk1) and a second pathway that was originally characterised on the basis of its involvement in yeast-hypha morphogenesis (defined by the MAPK Cek1) (Fig. 1). Both pathways are evolutionarily conserved in other fungi (Román et al., 2007).

The cell integrity pathway includes a MAPKK module that incorporates the MAPKKK Bck1, the MAPKK Mkk1 and the MAPK Mk1 (Alonso-Monge et al., 2006). Mk1 activation by cell wall stress is mediated through protein kinase C (Pkc1) signalling (Paravicini et al., 1996; Alonso-Monge et al., 2006). The disruption of Mk1 confers sensitivity to cell wall stresses and elevated temperatures (Navarro-García et al., 1995). Mk1 inactivation does not increase the sensitivity of C. albicans to killing by neutrophils or macrophages (Arana et al., 2007), but does attenuate the virulence of C. albicans (Diez-Orejas et al., 1997).

The morphogenetic MAPK (Cek1) pathway includes the MAPKKK Ste11, the MAPKK Hst7 and the MAPK Cek1 (Brown, 2002; Alonso-Monge et al., 2006). Components of this MAPK module are also involved in the C. albicans mating response (Chen et al., 2002), but Cek2 acts as the MAPK under these conditions. The Cek1 pathway is activated via the cell surface sensor Msb2 in response to cell wall damaging agents and mutations that affect cell wall integrity (Román et al., 2009; Cantero and Ernst, 2011). Inactivation of components on the Cek1 pathway inhibits filamentous growth under certain conditions and confers sensitivity to cell wall stresses (Leberer et al., 1996; Csank et al., 1998; Eisman et al., 2006; Cantero and Ernst, 2011). Candida albicans cek1 mutants are not hypersensitive to macrophage or neutrophil killing, but do display attenuated virulence (Csank et al., 1998; Arana et al., 2007).

**Oxidative stress**

*Candida albicans* is relatively resistant to reactive oxygen species (ROS), tolerating over 20 mmol l$^{-1}$ hydrogen peroxide (H$_2$O$_2$) under some conditions (Jamieson et al., 1996; Nikolau et al., 2009; Rodaki et al., 2009). This resistance is dependent on the AP-1-like transcription factor Cap1, which is an orthologue of *S. cerevisiae* Yap1 and *S. pombe* Pap1 (Alarco and Raymond, 1999), and upon the response regulator Skn7 (Singh et al., 2004) (Fig. 1). Cap1 contains redox-sensitive cysteine residues near its carboxy terminus that become oxidised following oxidative stress. This leads to the Hog1-independent nuclear accumulation of Cap1 and the activation of its target genes via Yap1-responsive elements (YRE) in their promoters (Zhang et al., 2000; Enjalbert et al., 2006; Znaidi et al., 2009). Cap1 targets include genes involved in the detoxification of oxidative stress (e.g. catalase and superoxide dismutase: *CAT1* and *SOD1*), glutathione synthesis (e.g. gamma-glutamylcysteine synthetase: *GCS1*), redox homeostasis and oxidative damage repair (e.g. glutathione reductase and thioredoxin: *GLR1* and *TRX1*). Together, these functions detoxify ROS and mediate cellular adaptation to stress. Consequently, the inactivation of Cap1 attenuates the induction of these genes, rendering *C. albicans* sensitive to oxidative stress (Alarco and Raymond, 1999; Enjalbert et al., 2006). The redox status of Cap1, and hence oxidative stress adaptation, is modulated by the redox regulator thioredoxin (Trx1) (da Silva Dantas et al., 2010).

The Hog1 MAPK pathway also contributes to oxidative stress resistance in *C. albicans* (Fig. 1). Inactivation of Hog1 and key upstream regulators confer oxidative stress sensitivity (Alonso-Monge et al., 2003; Chauhan et al., 2003; Smith et al., 2004; Kruppa and Calderone, 2006; da Silva Dantas et al., 2010; Smith et al., 2010). Oxidative stress signals appear to be transduced to Hog1 via the histidine kinases (Sh1, Chk1, Nik1), the response regulator Sks1 and the peroxiredoxin Ts1 (Kruppa and Calderone, 2006; Smith et al., 2010). An additional response regulator (Crr1) contributes to oxidative stress resistance in *C. albicans*, but is not required for Hog1 activation in response to H$_2$O$_2$ (Bruce et al., 2011).

Numerous observations indicate that *C. albicans* cells are exposed to oxidative stress during infection and that oxidative stress adaptation is essential for pathogenicity. There has been evolutionary expansion of the SOD gene family in *C. albicans*, with this pathogen carrying six superoxide dismutase genes. Transcript profiling experiments have demonstrated that oxidative stress genes are induced following exposure to host macrophages and neutrophils (Rubin-Bejerano et al., 2003; Lorenz et al., 2004; Fradin et al., 2005), and during mucosal infection (Zakikhany et al., 2007), but are not activated to the same extent during tissue infection (Thewes et al., 2007; Walker et al., 2009; Wilson et al., 2009). These expression patterns have been confirmed by single cell profiling of *C. albicans* cells tagged with diagnostic GFP fusions to oxidative stress genes (Enjalbert et al., 2007; Arana et al., 2007; Miramón et al., 2012). The inactivation of genes involved in ROS detoxification, such as superoxide dismutates and catalase, renders *C. albicans* cells more sensitive to phagocytic killing and attenuates the virulence of the fungus (Wysong et al., 1998; Hwang et al., 2002; Fradin et al., 2005; Frohner et al., 2009). Similar phenotypes are also observed following the perturbation of oxidative stress regulators. *Candida albicans* cap1 and hog1 mutants are killed more effectively by phagocytes (Fradin et al., 2005; Arana et al., 2007), and hog1 and trx1 mutants display attenuated virulence (Alonso-Monge et al., 1999; da Silva Dantas et al., 2010; Cheetham et al., 2011). Taken together, the data suggest that *C. albicans* exploits robust oxidative stress responses to protect itself from phagocytic killing, but these responses become less vital as the fungus develops systemic infections.

**Nitrosative stress**

Exposure to reactive nitrogen species (RNS), for example nitric oxide, causes molecular damage such as the S-nitrosylation of the thiol groups of cysteines in proteins and glutathione. RNS exert static rather than cidal effects on *C. albicans* (Kaloriti et al., 2012). *Candida albicans* responds to nitrosative stress by activating a defined set of genes that includes oxidative stress functions such as catalase (Cat1), glutathione-conjugating and -modifying enzymes, and NADPH oxidoreductases and dehydrogenases (Hromatka et al., 2005). In addition, *YHB1* expression is strongly induced. *YHB1* is one of three genes encoding flavohaemoglobin-related proteins in *C. albicans*: *YHB1*, *YHB4* and *YHB5* (Ullmann et al., 2004;
Tillmann et al., 2011). Of these, only YHB1 is induced in response to nitric oxide, and this gene encodes the major nitric oxide dioxygenase responsible for nitric oxide detoxification (Ullmann et al., 2004; Hromatka et al., 2005). Following RNS detoxification, redox homeostasis is restored and S-nitrosylated adducts are repaired, allowing C. albicans to resume growth (A.T. and A.J.P.B., unpublished).

Little is known about the signalling pathways that mediate the nitrosative stress response in C. albicans, or in other yeasts for that matter. However, it has been shown that the zinc finger transcription factor Cta4 is responsible for activating YHB1 expression in response to RNS (Chiranand et al., 2008), and the inactivation of either CTA4 or YHB1 confers nitrosative stress sensitivity (Ullmann et al., 2004; Chiranand et al., 2008) (Fig. 1).

In addition to ROS, the molecular armoury of phagocytic cells includes RNS that contribute to fungal killing (Rementeria et al., 1995; Vázquez-Torres and Balish, 1997; Brown, 2011). Not surprisingly, therefore, nitrosative stress genes are induced following phagocytic attack (Fradin et al., 2005; Zakikhany et al., 2007). Nitrosative stress genes are also upregulated during mucosal infections (Zakikhany et al., 2007). However, the response is not strongly activated during systemic infection (Thewes et al., 2007; Walker et al., 2009), and the inactivation of Yhb1 or Cta4 only causes a slight reduction in virulence in the mouse model of systemic candidiasis (Hromatka et al., 2005; Chiranand et al., 2008). Therefore, the nitrosative stress response seems to be most important during the early stages of infection when the fungus is battling with host immune defences.

Several common themes are apparent from this brief overview of key stress responses in C. albicans. First, these stress-signalling pathways include regulators that have been highly conserved during fungal evolution. Examples include the Hog1, Mek1 and Cek1 MAPK modules, and the transcription factors Hsf1 and Cap1. Second, in comparison with the benign model yeasts S. cerevisiae and S. pombe, these stress responses have been evolutionarily tuned to the types and intensities of stresses that C. albicans encounters during its interactions with the host. Third, these stress responses are intimately linked to the virulence of this pathogen (Brown et al., 2007; Brown et al., 2012a; Román et al., 2007).

Adaptation to sequential stresses
Almost without exception, all of the above studies on stress adaptation in C. albicans have examined the responses of cells to individual stresses following growth on glucose. Yet, as described above, this pathogen inhabits diverse, complex and dynamic niches in the host. In these niches C. albicans will be exposed to multiple stresses. At times these stresses may be imposed sequentially. At other times, multiple stresses are imposed simultaneously such that the fungus is exposed to ‘combinatorial stress’. Furthermore, as glucose is either limiting or absent from many host niches, C. albicans cells must adapt to these stresses whilst exploiting alternative carbon sources. Recent data have revealed that these factors significantly influence stress adaptation in C. albicans. This section addresses adaptation to sequential stresses, and the following section discusses the impact of combinatorial inputs upon stress adaptation.

With regard to sequential stresses, it has been known for some time that prior exposure to a non-lethal dose of a stress can protect yeast cells against a subsequent dose of that same stress (Fig. 2A). For example, acquired thermotolerance has been described in S. cerevisiae, S. pombe and more recently in C. albicans (De Virgilio et al., 1990; Piper, 1993; Argüelles, 1997). Acquired tolerance has also been observed for oxidative stress in C. albicans (Jamieson et al., 1996). Acquired stress tolerance is dependent upon the activation of a molecular response to the initial stress, which represents the induction and accumulation of key proteins or metabolites that mediate adaptation to that stress. These proteins and metabolites represent a ‘molecular memory’ that can then protect the cell against a subsequent stress, leading to increased survival. However, this molecular memory is transient (Leach et al., 2012c), with the length

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**Fig. 2. Acquired stress tolerance and stress cross-protection in yeasts.** (A) Prior exposure to a stress can protect C. albicans cells against subsequent exposure to that stress (acquired stress tolerance) (upper panel). This indicates the existence of a molecular memory (see ‘Adaptation to sequential stresses’). However, the molecules that represent this memory have biological half-lives. Therefore, this molecular memory is transient, and will be lost during protracted time intervals between stresses (lower panel). (B) In some yeasts, some stresses (stress 1; blue) activate a core transcriptional response (purple) that includes genes that protect against another stress (stress 2; red). In this case, prior exposure to stress 1 often activates a molecular memory that confers protection against stress 2 (upper panel). However, if this core transcriptional response does not include genes that protect against a third stress (stress 3; green), then prior exposure to stress 1 does not activate a relevant molecular memory and does not confer protection against stress 3 (lower panel).
of the memory depending upon the decay rates of these proteins and metabolites (Fig. 2A). For example, in the case of thermotolerance, the molecular memory in *C. albicans* probably represents HSPs and trehalose biosynthetic enzymes rather than the stress protectant trehalose (Argüelles, 1997; Leach et al., 2012c), because trehalose levels decline rapidly once *C. albicans* cells are returned to lower temperatures (Argüelles, 1997). For osmotolerance in *C. albicans*, the molecular memory is thought to be mediated by glycerol biosynthetic enzymes rather than the osmolyte glycerol (You et al., 2012), because glycerol is rapidly extruded from yeast cells when the osmotic stress is removed (Klipp et al., 2005). By analogy, acquired tolerance to oxidative stress is probably mediated by the accumulation of antioxidant enzymes rather than antioxidants themselves (Jamieson et al., 1996).

In some cases, prior exposure to a non-lethal dose of one type of stress can also protect yeast cells against a subsequent dose of a different type of stress—a phenomenon called stress cross-protection (Fig. 2B). For example in *S. cerevisiae*, a mild heat shock protects cells against a subsequent oxidative stress (Wieser et al., 1991; Lewis et al., 1995). Similarly, pre-treatment with an oxidative, osmotic or thermal stress promotes freeze–thaw tolerance in *S. cerevisiae* (Park et al., 1997). The molecular basis for this phenomenon lies in the core transcriptional response to stress whereby exposure to any one of several different types of stress activates genes involved in adaptive responses to many types of stress (Fig. 3). For example, in *S. cerevisiae* exposure to thermal, osmotic, oxidative or pH stress activates several hundred genes with roles in stress adaptation, central metabolism and energy generation (Gasch et al., 2000; Causton et al., 2001). This core stress response is largely dependent on the functionally redundant transcriptional activators Msn2 and Msn4, which bind to stress response elements in the promoters of their target genes to mediate their activation (Mager and De Kruijff, 1995; Gasch et al., 2000; Causton et al., 2001). Msn2 and its stress-induced transcriptional activation are downregulated by glucose via the cAMP-protein kinase A (PKA) signalling pathway (Görner et al., 1998; Garreau et al., 2000).

An analogous Msn2-dependent core transcriptional response to stress is displayed by *Candida glabrata*, which in evolutionary terms lies much closer to *S. cerevisiae* than to *S. pombe* or *C. albicans* (Roetzer et al., 2008) (Fig. 3). *Schizosaccharomyces pombe* also displays a core stress response. However, in this case the response is driven by Sty1 (Chen et al., 2003), which is the orthologue of the Hog1 SAPK in *S. cerevisiae*, *C. glabrata* and *C. albicans* (Nikolaou et al., 2009). In contrast, *C. albicans* was initially thought to lack a core transcriptional response to stress (Enjalbert et al., 2003). Subsequent work revealed that this yeast does display a core stress response, but one that comprises a much smaller subset of roughly 25 genes (Enjalbert et al., 2006). In *C. albicans* the roles of Msn2/4-like transcription factors have diverged significantly (Nicholls et al., 2004; Ramsdale et al., 2008), and the core stress response is coordinated by Hog1 and Cap1 (Enjalbert et al., 2006). Clearly there has been significant rewiring of the circuitry that regulates the core stress response, as well as of the response itself.

This has significant implications for the behaviour of *C. albicans* during exposure to sequential stresses. Thermal stress protects *C. albicans* against a subsequent oxidative stress, but not against a subsequent osmotic or cell wall stress (Enjalbert et al., 2003; Leach et al., 2012a). This cross-protection is dependent on Cap1 and correlates with the induction of some Cap1 target genes during heat shock (Nicholls et al., 2009; Leach et al., 2012a). However, this cross-protection is asymmetric, as an initial treatment with oxidative stress does not protect *C. albicans* cells against a subsequent thermal stress (Enjalbert et al., 2003; Leach et al., 2012a).

These observations are reminiscent of the phenomenon of microbial adaptive prediction (Mitchell et al., 2009) (Fig. 4A). Mitchell and co-workers argue that some microorganisms inhabit relatively predictable environments, in which one type of environmental change is often followed by a second type of stimulus. In such cases organisms may have evolved a regulatory circuitry that allows them to predict the second stimulus, thereby conferring an evolutionary advantage. This type of adaptive prediction is displayed by *S. cerevisiae*, which exploits the elevated temperatures associated with vigorous fermentation to induce oxidative stress genes that will be required once glucose is exhausted and cells switch to respiratory and oxidative metabolism (Mitchell et al., 2009). This adaptive prediction is asymmetric, as...
oxidative stress does not induce heat shock gene expression in *S. cerevisiae* (Mitchell et al., 2009). An analogous asymmetric relationship between oxidative and heat shock gene regulation is observed in *C. albicans*: in general, oxidative stress responses are induced in response to heat shock, but heat shock genes are not induced by an oxidative stress (Enjalbert et al., 2003) (Fig. 4B). This is consistent with the idea that adaptive prediction might have evolved in *C. albicans* such that the pathogen anticipates oxidative attack by phagocytic cells in response to fever associated with inflammatory responses.

A second example of adaptive prediction has been described in *C. albicans*. In this fungus, oxidative stress genes are activated following exposure to glucose, thereby conferring elevated resistance to acute oxidative stress (Rodaki et al., 2009) (Fig. 4B). This phenomenon does not depend on Hog1 or Cap1 (Rodaki et al., 2009). Instead, glucose-enhanced oxidative stress resistance appears to be regulated by evolutionarily conserved glucose signalling pathways (I.B. and A.J.P.B., unpublished). This anticipatory response, which is triggered by the glucose concentrations present in the bloodstream, is likely to be relevant in the disease context. *Candida albicans* cells that enter the bloodstream are exposed to glucose, and this may help to protect them against the impending attack by phagocytic cells. If this were true, the phenomenon of glucose-enhanced oxidative stress resistance must have evolved relatively recently. This appears to be the case (I.B. and A.J.P.B., unpublished). Indeed, the opposite phenotype is observed in *S. cerevisiae*: glucose reduces stress resistance in this benign yeast, and this may help to protect against the impending attack by phagocytic cells. If this were true, the phenomenon of glucose-enhanced oxidative stress resistance must have evolved relatively recently. This appears to be the case (I.B. and A.J.P.B., unpublished). Indeed, the opposite phenotype is observed in *S. cerevisiae*: glucose reduces stress resistance in this benign yeast, and this may help to protect against the impending attack by phagocytic cells. If this were true, the phenomenon of glucose-enhanced oxidative stress resistance must have evolved relatively recently. This appears to be the case (I.B. and A.J.P.B., unpublished). Indeed, the opposite phenotype is observed in *S. cerevisiae*: glucose reduces stress resistance in this benign yeast, and this may help to protect against the impending attack by phagocytic cells. 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This anticipatory response appears to be symmetric because exposing *C. albicans* cells to hydrogen peroxide leads to the activation of genes involved in central carbon metabolism (Enjalbert et al., 2006). However, this particular response (oxidative stress-induced metabolic activation), which is conserved in other yeasts (Gasch et al., 2000; Causton et al., 2001; Chen et al., 2003; Enjalbert et al., 2006), may have less to do with anticipatory prediction and more to do with the need for metabolic intermediates and energy to drive oxidative stress adaptation (Brown et al., 2012a).

**Adaptation to combinatorial stresses**
As mentioned above, *C. albicans* cells are often simultaneously exposed to multiple stresses within the complex host niches they inhabit. Possibly the best example of combinatorial stress occurs following phagocytosis by neutrophils or macrophages, when the fungus is bombarded with ROS, RNS and cationic fluxes (Rementeria et al., 1995; Vázquez-Torres and Balish, 1997; Brown, 2011; Nüsse, 2011). However, combinatorial stresses are likely to be relevant in many other host niches, such as during mucosal invasion (where oxidative stresses are encountered while adjusting cellular water balance) and kidney infection (where respiring cells must deal with endogenous ROS while adapting to relatively high salt concentrations). How do *C. albicans* cells respond to such combinatorial stresses? We have predicted that the adaptive responses to such combinatorial stresses might not be equivalent to the sum of the responses to the corresponding individual stresses (Kalariti et al., 2012). Our rationale is that unexpected cross-talk between the relevant signalling pathways might exist. Several examples of this have emerged recently.

Combinatorial oxidative (H$_2$O$_2$) plus nitrosative stresses (dipropylentetramine-NONOate, DPTA-NONOate) and combinatorial cationic (NaCl) plus nitrosative stresses appear to exert additive effects upon the growth of *C. albicans* cells (Kalariti et al., 2012). However, YHBI1 gene induction is attenuated under these conditions, indicating that Cta4 signalling is compromised (A.T. and A.J.P.B., unpublished). Significantly, non-additive effects are observed for combinatorial cationic plus oxidative stresses (Kalariti et al., 2012). These stresses kill *C. albicans* synergistically. The basis for this appears to be ‘stress pathway interference’, whereby both Cap1 and Hog1 signalling are compromised by the combination of cationic and oxidative stress. As a result, cationic and oxidative stress genes are not induced, and intracellular ROS levels increase, leading to cell death (D.K., M.D.J., A.T. and A.J.P.B., unpublished). Indeed, hydrogen peroxide has been shown to stimulate apoptotic cell death in *C. albicans* via Ras-cAMP signalling (Phillips et al., 2003; Phillips et al., 2006). This appears to be highly relevant to host–fungus interactions because the effective killing of *C. albicans* cells by human neutrophils appears
to depend on the extreme potency of combinatorial cationic plus oxidative stresses (D.K., M.D.J., A.T. and A.J.P.B., unpublished). Combinatorial effects are also observed between thermal and other stresses. For example, elevated temperatures decrease the sensitivity of \textit{C. albicans} cells to a cell wall stress (Calcofluor White), but have little effect upon osmo-sensitivity (Leach et al., 2012a). This stress interaction appears to be mediated via Hsp90 (Leach et al., 2012a). As described above, the Hog1, Mkc1 and Cek1 pathways modulate cell wall functionality. The MAP kinases in these pathways are all client proteins of Hsp90, and their activation is modulated by Hsp90 (Leach et al., 2012a). Temperature fluctuations have been shown to influence Hsp90 expression levels as well as the binding of Hsp90 to its client proteins in \textit{C. albicans} (Nicholls et al., 2009; Diezmann et al., 2012; Leach et al., 2012a; Leach et al., 2012c). Furthermore, ambient temperature affects the cell wall proteome, and Hsp90 depletion alters cell wall architecture (Leach et al., 2012a; Heilmann et al., 2013). Therefore, Hsp90 has been proposed to act as a biological transistor that tunes environmental responses, including cell wall remodelling, to the ambient temperature of the cell (Leach et al., 2012b).

Therefore, as predicted (Kaloriti et al., 2012), combinatorial stresses exert unexpected effects upon the classical regulatory pathways that mediate responses to specific stresses (Fig. 1). The available data have revealed several distinct molecular mechanisms by which combinatorial cross-talk can occur (Fig. 5). First, there appears to be signalling cross-talk between the MAPKs in critical stress signalling pathways. This is suggested by mutational analyses whereby the deletion of \texttt{HOG1} leads to the derepression of Cek1 phosphorylation and the inhibition of Mkc1 phosphorylation (Arana et al., 2005). Cross-talk also exists at the chemical level. Combinations of H$_2$O$_2$ and NaCl lead to the formation of hypochlorous acid (HOCl), and nitric oxide and superoxide react to form peroxynitrite (ONOO$^-$), and nitrite and hypochlorous acid combine to form nitril chloride (NO$_2$Cl), generating cocktails of toxic compounds that can damage lipids, proteins and nucleic acids (reviewed by Brown et al., 2009). We have now shown that combinatorial effects can also be triggered at the biochemical level. In this case the inhibition of key detoxification functions by cationic stresses leads to the build up of intracellular ROS, causing stress pathway interference and ultimately cell death (D.K., M.D.J., A.T. and A.J.P.B., unpublished). In addition, we have shown that combinatorial effects can be mediated by a biological transistor. In this case, Hsp90 coordinates the activities of multiple signalling pathways involved in cellular adaptation (Leach et al., 2012b). While the responses of fungal cells to individual stresses are now reasonably well understood, little is known about the mechanisms that underlie combinatorial stress adaptation. Yet, combinatorial stress adaptation is highly relevant to natural environments.

**Impact of dynamic host niches upon stress adaptation**

Metabolic changes within host niches also affect stress adaptation in \textit{C. albicans} (Fig. 6). In particular, many host niches either lack sugars such as glucose or contain glucose at low concentrations. Instead, these niches contain complex mixtures of alternative carbon sources such as amino acids, carboxylic acids such as lactate, and fatty acids. Consequently, \textit{C. albicans} must assimilate these alternative carbon sources if it is to grow and colonise these niches. Not surprisingly, metabolic pathways that are essential for the assimilation of these alternative carbon sources, such as gluconeogenesis and the glyoxylate cycle, are required for full virulence (Lorenz and Fink, 2001; Barelle et al., 2006; Piekarska et al., 2006; Ramirez and Lorenz, 2007). Furthermore, lactate assimilation is essential for \textit{C. glabrata} to colonise the intestine (Ueno et al., 2011), and a significant proportion of \textit{C. albicans} cells infecting the kidney activate pathways for alternative carbon utilisation (Barelle et al., 2006), as do phagocytosed \textit{C. albicans} cells (Lorenz et al., 2004; Fradin et al., 2005; Barelle et al., 2006; Miramón et al., 2012). The metabolic activity of \textit{C. albicans} can modify the pH of its microenvironment (Vylkova et al., 2011) adding to the dynamism of host niches. The regulatory circuitry that

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**Fig. 5. Mechanisms underlying combinatorial stress effects in \textit{C. albicans}.** Several distinct mechanisms contribute to combinatorial stress effects in \textit{C. albicans} (see ‘Adaptation to combinatorial stresses’). (A) Classical cross-talk occurs between the MAPK signalling pathways (Alonso Monge et al., 2006). Hog1 signalling pathway: Ssk2, MAPKKK; Pbs2, MAPK; Hog1, MAPK/SAPK. Cell integrity pathway: Bck1, MAPKK; Mkk1, MAPKK; Mkc1, MAPK. Mating/invasive growth pathway: Ste11, MAPKKK; Hst7, MAPKK; Cek1, MAPK. (B) Hsp90 acts as a biological transistor, modulating the activities of the transcription factor Hsf1 and the MAPKs in response to thermal fluctuations (Leach et al., 2012a; Leach et al., 2012b). (C) Combinatorial cationic plus oxidative stress leads to stress pathway interference, whereby Hog1 and Cap1 signalling are affected by oxidative and cationic stress, respectively (D.K., M.D.J., A.T. and A.J.P.B., unpublished). (D) There is cross-talk at the chemical level, whereby different reactive oxygen species (ROS), reactive nitrogen species (RNS) and reactive chlorine species (RCS) can be generated spontaneously and by enzymatic catalysis (Brown et al., 2009; Brown et al., 2011), presumably leading to the activation of different subsets of stress genes.
regulates carbon assimilation in *C. albicans* has undergone evolutionary rewiring (Ihmls et al., 2005; Martchenko et al., 2007; Lavoie et al., 2009; Sandai et al., 2012), just as is the case for stress adaptation (discussed above).

Despite the fact that glucose is limiting or absent in many host niches, most studies of stress adaptation in *C. albicans* have been performed on cells grown in media containing 2% glucose. Recently, we showed that growth on physiologically relevant alternative carbon sources, such as lactate or oleic acid, affects stress adaptation in *C. albicans* (Ene et al., 2012a). Lactate-grown cells are more resistant to osmotic stress, cell wall stresses and some antifungal drugs. This increased stress resistance is not dependent on Hog1 or Mkc1 signalling. Instead, it relates to the effects of alternative carbon sources on the proteomic content and architecture of the cell wall, which in turn impact upon the biophysical properties of the cell wall (Ene et al., 2012a; Ene et al., 2012b) (Fig. 6). These alterations at the cell surface affect host recognition of *C. albicans* cells and influence the virulence of this pathogen in both systemic and mucosal models of infection (Ene et al., 2012a; Ene et al., 2013). Clearly, metabolic adaptation affects stress responses in *C. albicans*, and this further complicates our understanding of environmental adaptation of this fungus within the complex and dynamic microenvironments it occupies during host colonisation and disease progression. Significantly, this is also likely to affect the efficacy of antifungal drug treatments against individual *C. albicans* cells in these niches (Ene et al., 2012a).

**Outlook**

Significant advances have been made in our understanding of stress adaptation in *C. albicans*, and progress is being made towards the elaboration of specific stress signalling pathways. This is important because stress adaptation contributes to the virulence of this major fungal pathogen of humans. However, host niches are complex and dynamic, and the impact of this complexity and dynamism upon stress adaptation remains largely unexplored. In particular, how are stress responses regulated temporally during host colonisation and disease progression? The elegant microarray studies performed by Bernie Hube’s group go some of the way to addressing this question (Fradin et al., 2005; Thewes et al., 2007; Zakikhany et al., 2007; Wilson et al., 2009). However, microarray studies average the molecular behaviour of the fungal population as a whole, and fungal populations display heterogeneous behaviours in host niches (Barelle et al., 2006). This is because the microenvironments of individual cells vary even within specific host niches. Therefore, the spatial regulation of stress adaptation must also be examined during infection. This must either be done by examining the responses of individual cells in vivo, for example using GFP-based single-cell profiling methods (Barelle et al., 2006; Enjalbert et al., 2007; Miramón et al., 2012), or by increasing the sensitivity of RNA sequencing technologies and increasing their spatial resolution, for example by exploiting laser capture microscopy. These approaches are being pursued by the Aberdeen Fungal Group (J.P., S.S. and A.J.P.B., unpublished).

In addition, at least three aspects of stress adaptation that are of direct relevance in vivo need further dissection in vitro. First, which anticipatory responses in *C. albicans* influence host colonisation and disease progression, and how are these anticipatory responses controlled at the molecular level? Second, which combinatorial stress responses in *C. albicans* influence host–fungus interactions, and how are they regulated? Third, how does metabolic adaptation influence stress resistance within host niches? Despite the limited exploration of these issues, it is already clear that they involve non-additive behaviours that reflect unexpected signalling, transcriptional, biochemical and chemical cross-talk. Furthermore, many of these responses are dynamic and dose dependent. Given their complexity, a combination of experimental approaches and predictive mathematical modelling seems especially important for the development of a true understanding of these adaptive processes. Such studies will provide important insights into the forces that have driven the recent evolution of this pathogen in its host.

In closing, it is worth emphasising that studies of stress adaptation are revealing points of fragility in *C. albicans* that could potentially provide targets for translational research directed towards the development of novel antifungal therapies. Indeed, the therapeutic potential of Hsp90 inhibitors is being pursued by a number of laboratories (Dolgin and Motluk, 2011). Therefore, observations such as the acute sensitivity of *C. albicans* towards combinatorial cationic plus oxidative stress could, in principle, be exploited therapeutically.

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