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REVIEW

The contribution of *Aspergillus fumigatus* stress responses to virulence and antifungal resistance

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Invasive aspergillosis has emerged as one of the most common life-threatening fungal disease of humans. The emergence of antifungal resistant pathogens represents a current and increasing threat to society. In turn, new strategies to combat fungal infection are urgently required. Fungal adaptations to stresses experienced within the human host are a prerequisite for the survival and virulence strategies of the pathogen. Here, we review the latest information on the signalling pathways in *Aspergillus fumigatus* that contribute to stress adaptations and virulence, while highlighting their potential as targets for the development of novel combinational antifungal therapies.

***Keywords*:** aspergillosis, osmotic stress, oxidative stress, cell wall stress, hypoxia, heat shock, fungicide resistance

Introduction

Currently, more people die from fungal infections than malaria and tuberculosis, which is in part due to the rise of individuals with compromised immunity (Brown *et al.*, 2012). Invasive aspergillosis (IA) has emerged as one of the most common life-threatening fungal diseases of immuno-compromised humans and has been reported to have mortality rates as high as 90% (Chauhan *et al.*, 2006). IA is predominantly caused by *Aspergillus fumigatus*, a ubiquitous environmental and air-borne pathogen, which is constantly inhaled by humans. Despite the increased frequency of life-threatening fungal diseases, a very limited number of antifungal drugs are at our disposal. Polyenes, azoles, and echinocandins are used to combat *A. fumigatus* infections. Sterol-chelating polyenes and Cyp51-inhibiting azoles target fungal cell membranes and ergosterol biosynthesis, while echinocandins are β-1,3-glucan synthase inhibitors that impact on fungal cell wall biosynthesis. Despite the long-term usage of polyenes, such as Amphotericin B, resistance remains rare. However, mutations in the *cyp51A* gene, which encodes a lanosterol 14 α-demethylase (Lelievre *et al.*, 2013) and the over-expression or mutation of the *fks1* β-1,3-glucan synthase encoding gene, represent common mechanisms for azole and echinocandin resistance in clinical *A. fumigatus* isolates (Rocha *et al.*, 2007; Arendrup *et al.*, 2009). The rise in the human population susceptible to IA and the emergence of antifungal resistant pathogens presents a current and increasing threat to society. In turn, new strategies to combat fungal infections, are urgently required.

The ability to sense and adapt to your surroundings is essential for fungal survival in a heterogeneous environment. Fungal adaptations to stresses experienced within the human host are a prerequisite for the survival and virulence strategies of the pathogen (Gasch *et al.*, 2007). Although this defines a more general aspect of pathogenicity, the increased understanding of such fundamental mechanisms could prove key to the development of novel, and combinational, strategies to combat fungal infections. Our increased awareness of fungal stress tolerance and pathogenesis has been facilitated by the development of functional genomics, bioinformatics tools and reverse-genetic approaches to identify and study gene function. Here, we review the latest information on the signalling pathways in *A. fumigatus* that contribute to stress adaptations and virulence with the view of highlighting potentially novel antifungal therapies (Table 1). Primarily, we will describe the stresses experience by *A. fumigatus*, within the mammalian host, during the establishment of invasive pulmonary aspergillosis.

Stress exposure within a mammalian host

Once inhaled into the lungs of a mammalian host, the conidia and subsequently the mycelia of *A. fumigatus* are exposed to a variety of stresses (Fig. 1). Innate immunity is crucial for protection against IA and following inhalation by a human *A. fumigatus* conidia are phagocytised by alveolar macrophages and killed by the production of reactive oxygen species (ROS). Germination promotes neutrophil recruitment and the increased release of ROS to kill the mycelia (Chauhan *et al.*, 2006). Most fungi have evolved to grow at ambient temperatures, while *A. fumigatus*, which is considered a meso-

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Table 1. The influence of *Aspergillus fumigatus* stress response pathways on stress tolerance and pathogenicity. The reported resistance to exogenous stresses and fungal virulence in a model of pulmonary aspergillosis was assessed post genetic disruption of the respective protein.

Signalling pathway	Fungal protein	Impact of absence of fungal protein on			Reference
		Stress tolerance	Antifungal sensitivity	Virulence	
Cell wall integrity (CWI)	PkcA	Increased sensitivity to oxidative stress (paraquat, menadione) and cell wall stress (calcofluor white, congo red). Thermo-tolerance decreased.	Increased sensitivity to voriconazole and caspofungin	Virulent	Rocha *et al.* (2015)
	Mkk2	Increased sensitivity to cell wall stress (calcofluor white, congo red) and alkaline pH. Thermo-tolerance decreased.	Increased sensitivity to posaconazole and voriconazole	Attenuated	Dirr *et al.* (2010)
	MpkA	Increased sensitivity to oxidative stress (menadione) and cell wall stress (calcofluor white, congo red). Thermo-tolerance decreased.	Increased sensitivity to caspofungin	Virulent	Valiante *et al.* (2008)
	SitA	Increased sensitivity to cell wall stress (calcofluor white, congo red).	na	Avirulent	Bom *et al.* (2015)
High osmolarity glycerol (HOG)	TscC (NikA)	Increased sensitivity to osmotic stress (sorbitol, mannitol, NaCl, KCl) and hypoxia. Increased resistance to cell wall stress (calcofluor white, congo red). Increased sensitivity to elevated calcium levels.	Increased resistance to micofungin, fludioxonil, iprodione, and pyrrolnitrin	Virulent	McCormick *et al.* (2012) Hagiwara *et al.* (2013)
	SskB	Increased sensitivity to osmotic (NaCl) and oxidative stress (paraquat).	Increased resistance to fludioxonil and iprodine	Attenuated	de Castro *et al.* (2014a)
	SakA (HogA)	Increased sensitivity to oxidative (H_2O_2, menadione) and osmotic stress. Increased sensitivity to elevated calcium levels. Increased sensitivity to benzoic acids.	na	na	Xue *et al.* (2004), Kim *et al.* (2010), de Castro *et al.* (2014a)
	PtcB	Increased sensitivity to cell wall stress (calcofluor white, congo red).	na	Avirulent	Winkelströter *et al.* (2015a, 2015b)
Calcium-Calcineurin	CnaA (CalA)	na	Loss of caspofungin paradoxical effect	Avirulent	Steinbach *et al.* (2006), Fortwendel *et al.* (2010)
	CrzA	Thermo-tolerance decreased.	Loss of caspofungin paradoxical effect Increased sensitivity to fludioxonil and iprodine	Avirulent	Soriani *et al.* (2008), de Castro *et al.* (2014a), Fortwendel *et al.* (2010)
Cross-pathway control (CPC)	CpcC	Increased sensitivity to amino acid starvation.	na	Virulent	Sasse *et al.* (2008)
	CpcA	Increased sensitivity to amino acid starvation.	na	Attenuated	Krappmann *et al.* (2004)
Unfolded protein response (UPR)	IreA	Increased sensitivity to cell wall stress (calcofluor white). Thermo-tolerance decreased. Impaired carbon and iron source utilisation.	Increased sensitivity to itraconazole and voriconazole	Avirulent	Feng *et al.* (2011)
	HacA	Increased sensitivity to oxidative stress (paraquat) and cell wall stress (calcofluor white). Thermo-tolerance decreased. Impaired nutrient assimilation from complex sources.	Increased sensitivity to caspofungin	Attenuated	Richie *et al.* (2009)
Target of rapamycin (TOR)	RhbA	Impaired growth on poor nitrogen sources. Increased sensitivity to rapamycin.	na	Attenuated	Panepinto *et al.* (2003)
Nitrogen catabolite repression	AreA	Impaired nitrate utilisation.	na	Attenuated	Hensel *et al.* (1998)
Heatshock	Hsp90	Increased sensitivity to cell wall stress (congo red) and inhibitors of calcineurin (FK506).	Increased sensitivity to caspofungin	na	Lamoth *et al.* (2012)
Reductive iron assimilation	FreB	Increased sensitivity to iron starvation.	na	na	Blatzer *et al.* (2011b)
	FtrA	na	na	Virulent	Schrettl *et al.* (2004)
Siderophore-mediated iron uptake	HapX	Increased sensitivity to iron starvation.	na	Attenuated	Schrettl *et al.* (2010)
	SidA	Increased sensitivity to iron starvation and oxidative stress (H_2O_2).	na	Avirulent	Schrettl *et al.* (2004)
	SidC	Increased sensitivity to iron starvation and oxidative stress (H_2O_2).	na	Attenuated	Schrettl *et al.* (2007)
	SidD	Increased sensitivity to iron starvation and oxidative stress (H_2O_2).	na	Attenuated	Schrettl *et al.* (2007)
	SidF	Increased sensitivity to iron starvation and oxidative stress (H_2O_2).	na	Attenuated	Schrettl *et al.* (2007)
Alkaline pH response	PacC	Increased sensitivity to alkaline pH.	Increased sensitivity to caspofungin	attenuated	Bertuzzi *et al.* (2014)
General stress response	SebA	Increased sensitivity to oxidative stress (H_2O_2, paraquat). Thermo-tolerance decreased. Impaired growth on poor nutrient sources.	na	Attenuated	Dinamarco *et al.* (2012a)
Sterol sensing	SrbA	Increased sensitivity to iron starvation and hypoxia.	Increased sensitivity to fluconazole	Attenuated	Blatzer *et al.* (2011a)
	SrbB	Increased sensitivity to hypoxia.	na	Attenuated	Chung *et al.* (2014)

phile, displays optimal growth at 37°C and is thermo-tolerant up to 50°C (Robert and Casadevall, 2009). This is proposed to be advantageous within the host during febrile conditions (Robert and Casadevall, 2009). Additional ambient conditions are also proposed to influence the survival of the pathogen within the host, including environmental pH (Bertuzzi *et al.*, 2014), oxygen availability (Wezensky and Cramer, 2009), and altered nutrient acquisition (Dagenais and Keller, 2009), while the administration of haematogenous antifungal drugs presents a novel stressors. The capacity of *A. fumigatus* to sense and respond to the aforementioned stressors is therefore fundamental to pathogenicity and antifungal tolerance.

Coping with nutrient limitation

Within the host environment *A. fumigatus* must adapt to, and assimilate, host-derived sources of carbon, nitrogen, and other essential elements such as iron. Autophagy recycles cellular components aiding the survival of nutrient limitation and is therefore marker for cellular starvation. Autophagy is regulated by TOR (target of rapamycin) signalling during nutrient, in particular nitrogen, limitation. The absence of the autophagy regulating kinase, Atg1, severely disrupts autophagy, yet has no impact upon virulence suggesting that autophagy does not play a major role in infection or there are alternative mechanisms to adapt to starvation (Richie *et al.*, 2007). In contrast, disruption of RhbA, which is a Ras-related Rheb protein involved in nitrogen utilisation and TOR signalling, displayed increased sensitivity to rapamycin, reduced growth rates on poor nitrogen sources and attenuated virulence (Panepinto *et al.*, 2003). Additionally, the absence of AreA transcription factor involved in nitrogen catabolite repression and nitrate utilisation also showed attenuated virulence (Hensel *et al.*, 1998). Hence, the adaptation of nitrogen assimilation appears to be required for full virulence. The maintenance of amino acid homeostasis may also be key to the survival of the pathogen within the host, as disruption of the cross-pathway control (CPC) mechanism via the deletion of the pathway-specific transcription factor, CpcA, increased sensitivity to amino acid starvation and caused attenuated virulence (Krappmann *et al.*, 2004). However, the absence of the upstream regulatory eIF2α kinase, CpcC, which increases sensitive to amino acid starvation, did not impact on virulence (Sasse *et al.*, 2008) suggesting that amino acid homeostasis is not perturbed during host infection, while a basal level of CPC activation is required. Mutants lacking specific enzymes required for the glyoxylate cycle retained full virulence, suggesting that fatty acids and lipids do not represent a major source of carbon during infection (Schöbel *et al.*, 2006). The methylcitrate cycle prevents the accumulation of the toxic propionyl coenzyme A during amino acid utilisation as a nutrient source. Mutation of the methylcitrate synthase reduced growth and secondary metabolite production, while increasing susceptibility to macrophage killing and reducing virulence, suggesting that *A. fumigatus* utilises native or host protein degradation as a source of nutrition within the host (Ibrahim-Granet *et al.*, 2008). Therefore, a proper response to low-quality nitrogen, altered carbon assimilation and potentially reduced amino acid sources appear to be integral to survival within the host.

Iron is essential for all eukaryotes and is indispensible for respiration, amino acid metabolism, DNA synthesis and sterols, while excess iron can cause harm via inducing the formation of ROS, yet the detoxification of ROS and the prevention of oxidative stress also depends on iron (Schrettl and Haas, 2011). Therefore, a coordinated systems to acquire, use and store iron is required. The mammalian immune system restricts iron availability to fungal invaders via the production of transferrin or lactoferrin, while adapted pathogens have evolved counter mechanisms to acquire host iron (Schrettl and Haas, 2011). *A. fumigatus* lacks a mechanism to specifically acquire iron from host heme, ferritin or transferrin, but instead utilises a low affinity ferrous iron and two high affinity, reductive iron assimilation (RIA) and siderophore-mediated, uptake systems. The *A. fumigatus* low affinity system has not been molecularly characterised, while the high affinity systems have gained significant attention. The plasma membrane bound ferrireductase commences RIA via reducing ferric iron into the more soluble ferrous form, which is re-oxidised and imported by the FetC ferroxidase – FtrA permease complex. Genetic disruption of the RIA system does not impact on *A. fumigatus* virulence (Schrettl *et al.*, 2004). *A. fumigatus* excretes two iron-chelating siderophores, fusarinine C, and triacetylfusarinine C, which are taken up by siderophore iron transporters. Within the fungus, the siderophores are partially hydrolysed by the EstB esterase, releasing the iron. Two additional intracellular siderophores, ferricrocin, and hydroxyferricrocin, are involved in iron storage (Schrettl and Haas, 2011). The biosynthesis of all siderophores starts with SidA-mediated hydroxylation of ornithine, which then branches into the biosynthesis of either the intra- or extra-cellular forms. Inhibiting the pro-

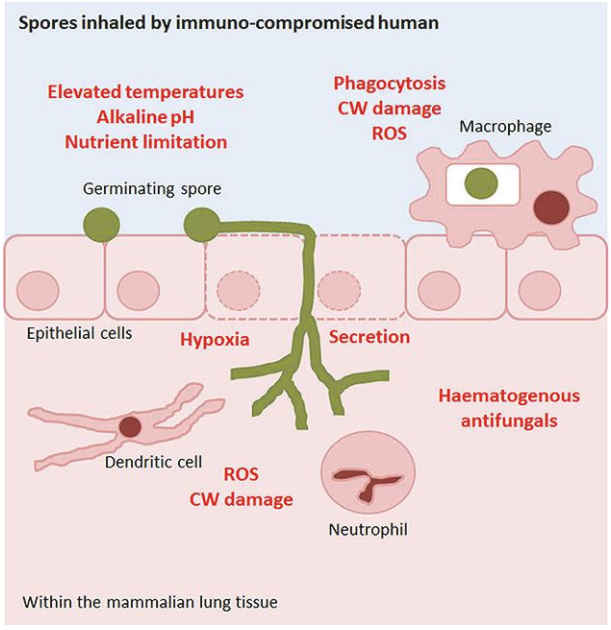

Fig. 1. ***Aspergillus fumigatus*** **successfully mitigates the impact of multiple stressors (red) during the colonisation of the mammalian host and the establishment of pulmonary invasive aspergillosis.**

duction of both forms of siderophores, via the Δ*sidA* mutation, renders *A. fumigatus* avirulent (Schrettl *et al.*, 2004), while defects in individual siderophore system, generated via the Δ*sidC*, Δ*sidD*, or Δ*sidF* mutations, only partially attenuated virulence, while decreasing fungal growth and survival within host macrophages (Schrettl *et al.*, 2007). Therefore, the regulation of siderophore production is fundamental to colonisation of the host environment, implicating adaptations to iron limitation.

Iron homeostasis and siderophore production is mediated by two counteracting transcription factors, SreA which represses RIA and the siderophore system during iron sufficiency in order to avoid toxic effects (Schrettl *et al.*, 2008) and HapX which represses iron-consuming pathways such as heme biosynthesis, respiration and ribosome biogenesis during iron starvation to spare iron (Schrettl *et al.*, 2010). A deficiency in HapX reduced siderophore biosynthesis and caused an attenuation of virulence, while the disruption of SreA had no impact on infection (Schrettl *et al.*, 2008, 2010), confirming that *A. fumigatus* experiences iron limitation during infection. Iron starvation induces iron acquisition and represses iron-dependent pathways such as respiration and the TCA cycle (Schrettl *et al.*, 2010), linking iron availability to carbon metabolism. The positive regulator of gluconeogenesis, AcuM, is well-known for a role in the regulation of genes involved in gluconeogenesis and the TCA cycle (Hynes *et al.*, 2007). Accordingly, the *ΔacuM* mutant was defective in gluconeogenesis. However, analyses of the *ΔacuM* transcriptome during growth on RPMI medium, reflecting the poor nutrient conditions within the host, revealed an enrichment of genes related to carbon metabolism and the synthesis of methionine and glutamate, in addition to iron homeostasis (Lui *et al.*, 2010). Subsequently, AcuM was shown to activate the expression of HapX and represses SreA, while the Δ*acuM* mutant was defective in growth and extracellular siderophore production during iron limitation, traits which accumulate in an attenuation of virulence (Lui *et al.*, 2010). As a consequence of the central role played by iron within the cell, the HapX-mediated iron limitation stress response is interlinked to primary metabolism, oxidative stress and virulence.

Maintaining cell wall integrity

The *A. fumigatus* cell wall provides the cell with structure and protection from environmental stresses. In this dynamic complex α-1,3-glucan, galactosaminogalactan, melanin and the RodA hydrophobin minimize exposure of the pathogen-associated molecular pattern, β-1,3-glucan, and host immune activation (Valiante *et al.*, 2015). Additionally, α-1,3-glucan, galactosaminogalactan and melanin are also involved in fungal adhesion to surfaces and biofilm formation, a growth form that enhances stress tolerance and antifungal resistance (Valiante *et al.*, 2015), while melanin provides increased ROS tolerance and promotes survival post phagocytosis (Jahn *et al.*, 1997). The absence of enzymes involved in the biosynthesis of the aforementioned cell wall components result in attenuated virulence (Beauvais *et al.*, 2014). Hence, the cell wall is essential to the ability of *A. fumigatus* to adapt to, and cope with, environmental changes within the host and the host's immune system. In response to cell wall stress, the cell wall integrity (CWI) pathway is activated. Protein kinase C (PkcA) commences the phosphorelay along the three sequential mitogen-activated protein kinases (MAPKs), Bck1, Mkk2 and MpkA (Fig. 2). The phosphorylated form of MpkA subsequently regulates the expression of genes involved in cell wall biosynthesis (Jain *et al.*, 2011). Additionally, MpkA influences the expression of genes involved in a general stress response, involving ROS tolerance and the production of

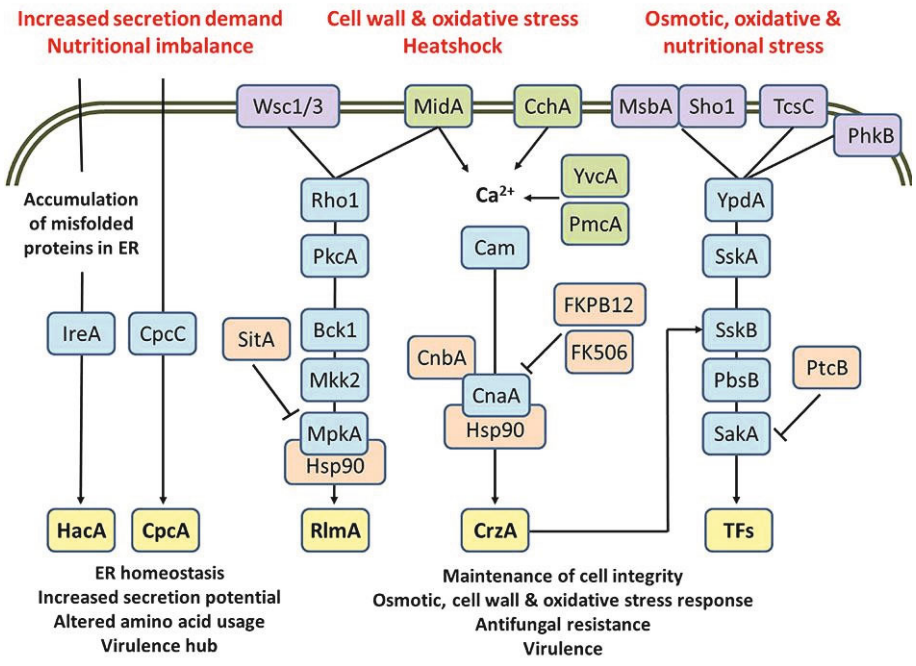

Fig. 2. The *Aspergillus fumigatus* stress response pathways that maintain fungal cell integrity, promoting virulence and antifungal resistance. Legend: purple, sensors; green, channel/transporter; blue, stress response pathway; yellow, transcription factor; orange, repressor of stress response signalling pathway.

siderophores, gliotoxin and melanin (Müller *et al.*, 2012). Disruption of the MAPKs cascade, or the upstream PkcA, increased fungal sensitivity to cell wall stresses, plus echinocandin and azole fungicides (Valiante *et al.*, 2009; Rocha *et al.*, 2015). However, the role of the CWI pathway in virulence remains of debate, as both the Δ*mpkA* and the *pkcA*^{G579R} mutants retained full virulence (Valiante *et al.*, 2008; Rocha *et al.*, 2015), while virulence of the intermediary Δ*mkk2* mutant was attenuated (Dirr *et al.*, 2010). The inconclusive evidence of the importance to the CWI pathway to virulence in *A. fumigatus* may reflect the interconnected and overlapping nature of MAPK signalling and the existence of compensation mechanisms.

The *A. fumigatus* high osmolarity glycerol (HOG), or stress-activated kinase, pathway consists of a two-component system (TCS) and MAPK cascade, terminating in SakA (HogA) phosphorylation (Fig. 2). Mutation of the TcsC (NikA) histidine kinase increases sensitivity to hyperosmotic stress and resistance to certain fungicides, such as fludioxonil. Exposure to hyperosmotic or fludioxonil stress results in the TcsC-dependent phosphorylation of SakA (McCormick *et al.*, 2012). SakA is also involved in conidial germination under nitrogen and carbon source starvation, plus the response to hypertonic conditions, ROS and heat shock (Xue *et al.*, 2004). The impact of SakA on virulence has not been assessed. However, the upstream MAPKK, SskB, is involved in the osmotic stress response and is required for full virulence (de Castro *et al.*, 2014a). In addition, the PtcB phosphatase has been shown to regulate SakA phosphorylation state and expression of osmo-dependent genes. The Δ*ptcB* mutant was more sensitive to cell wall damaging agents, had increased chitin and β-1,3-glucan, and impaired biofilm formation. The Δ*ptcB* strain was also avirulent (Winkelströter *et al.*, 2015a). Therefore, similar to the CWI pathway, the HOG pathway appears to influence cell wall composition, fungal development, antifungal resistance and pathogenicity, in addition to osmotic stress tolerance.

The calcineurin-calcium signalling pathway plays a multitude of roles in fungal biology, including a contribution to the maintenance of cell wall integrity, hyphal growth, antifungal resistance and virulence in *A. fumigatus* (Steinbach *et al.*, 2006; da Silva *et al.*, 2007; Juvvadi *et al.*, 2014). The absence of the calcineurin phosphatase or pathway-specific transcription factor, CrzA, impacts on calcium homeostasis, causing severe growth defects and attenuated virulence (Cramer *et al.*, 2008; Soriani *et al.*, 2008). Calcium homeostasis is maintained by calcium channels, pumps and transporters. Both the CchA voltage-gated channel, which senses membrane potential, and the MidA stretch-activated channel that is associated with contact sensing and thigmotropism, regulate calcium influx. Alternatively, vacuolar membrane localized YvcA channel mediates the release of calcium from the vacuole in response to hypertonic shock. Disruption of the three calcium channels demonstrated their involvement in calcium homeostasis, while the *cchA, midA*, and *yvcA* mutants were avirulent (de Castro *et al.*, 2014b). Therefore, high affinity calcium transport and the release of vacuolar calcium stores are essential for full virulence. Conversely, the vacuolar calcium ATPases, PmcA-C, are induced by elevated calcium levels in a CrzA-dependent manner and are involved in removing calcium from the cytosol. Disruption of PmcA had no impact on cell wall integrity or antifungal resistance, but did increase intracellular calcium levels, resulting in avirulence (Dinamarco *et al.*, 2012b). Therefore, the maintenance of calcium homeostasis is essential to infection.

The calcineurin-CrzA pathway appears to be central to numerous stress responses and CrzA is translocated to the nucleus post exposure to stress (Fig. 2). At increased concentrations, echinocandin fungicides, which inhibit β-1,3-glucans biosynthesis, become less effective due to the compensatory increase in cell wall chitin, termed the paradoxical effect. Similarly, the absence of calcineurin increases chitin content in response to reduced β-1,3-glucans. Therefore, compensatory mechanisms linking these two cell wall components represent a stress response. Calcineurin influences the transcription of cell wall biosynthetic gene *fksA*, which correlates with the reduction in β-1,3-glucans in the Δ*cnaA* mutant (Cramer *et al.,* 2008). Therefore, calcium signalling appears to overlap with MAPK pathways, influencing cell wall integrity. Chromatin immuno-precipitation DNA sequencing was used to explore the CrzA regulon identifying 102 directly regulated genes, including the PhkB histidine kinase and the SskB MAPKK of the TCS-HOG pathway (de Castro *et al.*, 2014b). In accordance, CrzA is translocated to the nucleus and is important for SakA phosphorylation in response to osmotic stress, while mutations to members of the TCS-HOG pathway render the respective strains more sensitive to high calcium levels. In addition, the Δ*sskB* mutant was avirulent and showed increased sensitive to oxidative stress (de Castro *et al.*, 2014a). These results show a link between calcium signalling and HOG pathway that is essential for full virulence. Therefore, a complex network of interconnected CWI, TCS-HOG, and calcineurin-CrzA signalling pathways coordinates the maintenance of the integrity of the fungal cell during host infection.

Nullifying oxidative stress

Following immune activation, host macrophages, neutrophils and other phagocytic cells produce high levels of ROS, which are toxic to fungal invaders (Brown *et al.*, 2009). Mice defective in ROS production are increasingly susceptible to *A. fumigatus* infections (Philippe *et al.*, 2003). To circumvent the function of phagocytes in the blood, alveoli, or at mucosal surfaces, *A. fumigatus* must induce the production of detoxifying antioxidants, a process that involves several stress response pathways. Melanin performs a multifaceted role in *A. fumigatus* including preventing immune-detection and protecting against UV light, lysis, in addition to oxidative stress, making the pigment fundamental to virulence (Hienekamp *et al.*, 2013). Defects in melanin biosynthesis cause a substantial increase in sensitivity to ROS (Hienekamp *et al.*, 2013). Transcriptomic studies revealed that the *A. fumigatus* oxidative stress response induces the expression of enzymes including catalases, superoxide dismutases and enzymes of the thioredoxin and glutathione pathways (Frealle *et al.*, 2013). Multiple genes involved in defence against ROS in *A. fumigatus* have been characterised. *A. fumigatus* possesses enzymes for ROS detoxification, including five catalases (*catA*,

cat1, *cat2*, *catC*, and *catE*) plus four superoxide dismutases (*sod1-4*). The absence of the condial catalase, *catA*, increased susceptibility to H_2O_2, while the absence of the hyphal catalases, *cat1* and *cat2*, did not. The individual loss of a single catalase did not impact on virulence. However, the simultaneous absence of *cat1* and *cat2* caused a reduction in virulence (Calera *et al.*, 1997; Paris *et al.*, 2003). In *A. fumigatus, sod1* and *sod2* were highly expressed in conidia, while *sod3* was strongly expressed in mycelia (Lambou *et al.*, 2010). The expression of *sod4* was low compared to other SODs, yet deletion of *sod4* was lethal (Lambou *et al.*, 2010). The absence of *sod1* or *sod2* caused increased sensitivity to ROS, while the loss of *sod3* only slightly delayed growth at high temperatures. Despite, the triple Δ*sod1* Δ*sod2* Δ*sod3* mutant showing increased sensitivity to ROS and killing by alveolar macrophages, no impact on virulence was observed (Lambou *et al.*, 2010). Therefore, the variable impact of multiple members of the oxidative stress response suggests a significant level of redundancy exists, potentially safeguarding functionality.

The Sho1 transmembrane protein which relays osmotic stress signals via the HOG pathway is also involved in oxidative stress tolerance, polarised growth and hyphal branching. However, the absence of Sho1 does not have an impact on virulence (Ma *et al.*, 2008). The downstream MAPK, SakA, has also been demonstrated to be involved in oxidative stress resistance, but its contribution to virulence is not known. Similarly the CWI pathway influences oxidative stress tolerance in *A. fumigatus*, with defects in PkcA and MpkA causing increased sensitivity to ROS, yet the Δ*mpkA* and the *pkcA*^{G579R} mutants remained virulent (Valiante *et al.*, 2009; Rocha *et al.*, 2015). Therefore, these two MAPK cascades are important for the oxidative stress response, yet it is not clear whether this specific role is essential to virulence (Fig. 2). The ROS sensing transcription factor Yap1 regulates the thioredoxin antioxidant pathway (Leal *et al.*, 2012) and protects against neutrophil killing, but is not required for full virulence (Lessing *et al.*, 2007). In *S. cerevisiae* the Msn2 and Msn4 transcription factors mediate a general stress response that functions in parallel to Yap1 (Hassan *et al.*, 2002). In *A. fumigatus*, a strain lacking the single orthologous transcription factor, SebA, showed increased sensitivity to oxidative stress and heat shock, plus growth defects under nutrient-poor conditions (Dinamarco *et al.*, 2012a). Accordingly, SebA accumulated in the nucleus upon exposure to oxidative stress and heat shock, while genes involved in the oxidative stress or heat shock response were regulated by SebA. Killing of the Δ*sebA* mutant by alveolar macrophages was increased, which corresponded to an attenuation of virulence. Therefore, SebA appears to be integrated in multiple stress responses, including oxidative stress, contributing to the survival of *A. fumigatus* within the host.

Heat shock and much more

Exposure to elevate temperatures is coupled with entry into a host organism and this is exaggerated by a febrile state. Despite temperature being a well-known factor to influence fungal development, little is known of how temperature sensing impacts on cell signalling. Hsp90 is an essential ATP-dependent molecular chaperone involved in protein folding, transport, maturation and degradation, which regulates the function of many central cell signalling proteins via altering the ratio of active to inactive protein (Cowen and Lindquist, 2005). Stresses, including elevated temperatures, increase the demand for Hsp90 compromising function and releasing the sequestered protein to become active. Additionally, Hsp90 may stabilise silent genetic variation, which is released in response to stress (Cowen and Lindquist, 2005). Therefore, Hsp90 regulates stress-signalling and promotes genetic variation, which enables fungal adaptations to stress. For example, the genetic or pharmacological inhibition of Hsp90 blocks the rapid evolution of antifungal resistance and enhances echinocandin activity against resistant infections (Cowen *et al.*, 2009). Therefore, Hsp90 has a diverse influence on fungal biology and evolution.

The potential for Hsp90 to interact with numerous proteins may correlate with Hsp90 having a wide ranging influence on multiple stress responses. Hsp90 is required for hyphal growth and proliferation, while Hsp90 inhibition caused increased sensitivity to the FK506 calcineurin inhibitor (Lamoth *et al.*, 2012) suggesting the two pathways are linked. The calcineurin-CrzA pathway is involved in the echinocandin-induced cell wall stress response in *A. fumigatus* (Steinbach *et al.*, 2007a). Hsp90 physically interacts with calcineurin catalytic subunit CnaA (Fig. 2). Hence, defects in the calcineurin-CrzA pathway phenocopy the impact of Hsp90 inhibition (Steinbach *et al.*, 2007b; Cowen, 2008). Neither temperature nor sub-inhibitory concentrations of Hsp90 inhibitor, geldanamycin, had a dramatic influence on fungal growth. However, eight *A. fumigatus* phosphatases, with putative roles in osmotic, oxidative and cell wall stress tolerance, also showed increased sensitivity to geldanamycin (Winkelströter *et al.*, 2015b). Therefore, it is probable that the influence of Hsp90 in numerous stress responses has only just begun to emerge and further investigations are required to determine which signal transduction pathways are jointly affected by Hsp90 and the identified phosphatases.

Hypoxia and sterol sensing

Oxygen is critical for obligate aerobes such as *A. fumigatus.* Necrotic lesions within the mammalian lungs are accompanied with decreased perfusion and low oxygen concentrations. Sites of hypoxia have been visualised during *A. fumigatus* infection, while metabolomic studies revealed the presence of ethanol in infected lungs, suggesting that *A. fumigatus* adapts to the low oxygen environments by switching to anaerobic fermentation (Grahl *et al.*, 2011). Transcriptional profiling of *A. fumigatus* during hypoxia revealed the up-regulation of genes involved in ergosterol biosynthesis and cell wall maintenance, while showing overlap with genes induced by iron limitation. Mutations in metabolic pathways involved in the hypoxia response impacted upon the ability of *A. fumigatus* to tolerate hypoxic micro-environments and iron limitation. Cellular sterol levels are tightly linked with oxygen availability. The sterol regulatory element-binding proteins (SREBP) regulate lipid metabolism in a range of organisms. Intriguingly, the expression of the *A. fumigatus*

SREBP orthologue, *srbA*, is induced by both hypoxia and iron starvation, which subsequently regulates genes involved in ergosterol biosynthesis, such as *erg11A/cyp51A*, plus RIA and siderophore biosynthesis including *fetC*, *ftrA*, *hapX*, and *sit1* (Willger *et al.*, 2008; Blatzer *et al.*, 2011a). Accordingly, the Δ*srbA* mutant was defective in hypoxia adaptations, growth under iron starvation, ergosterol biosynthesis, siderophore production and azole fungicide tolerance, while demonstrating highly attenuated virulence (Willger *et al.*, 2008; Blatzer *et al.*, 2011a). Subsequently, the HMG-CoA reductase that is required for ergosterol biosynthesis and the production of mevalonate for extracellular siderophore biosynthesis was shown to be regulated by SrbA, demonstrating the enzymatic link between the hypoxia, iron assimilation and the ergosterol pathway. Recently, an additional SREBP protein, SrbB, was also shown to co-regulate genes involved in heme biosynthesis and demethylation of C4-sterols during hypoxia, while independently regulating carbohydrate metabolism. Similar to the Δ*srbA*, the absence of SrbB attenuated virulence (Chung *et al.*, 2014). Therefore, hypoxia appears to prompt adaptations to iron limitation, while regulators of both the hypoxia and iron limitation responses mediate ergosterol biosynthesis, linking distinct mechanisms that *A. fumigatus* deploys to cope with the environmental conditions encountered within the host.

Adapting to alkaline pH

Transcriptomic analyses of pulmonary IA of a mouse showed the up-regulation of more than 100 alkaline-responsive genes (McDonagh *et al.*, 2008) implicating a role for pH-responsive cell signalling in adaptation to the alkaline host environment. The PacC transcription factor, which is highly conserved in fungi, regulates the expression of alkaline responsive genes (Peñalva *et al.*, 2008). The absence of PacC caused the impairment of growth at pH 8.0. The Δ*pacC* mutant was highly sensitive to cell wall targeting antifungals, and showed highly attenuated virulence potentially due to its non-invasive growth form caused by defects in epithelial entry and protease expression (Bertuzzi *et al.*, 2014). Therefore, pH-dependent PacC signalling influences traits such as host penetration and antifungal tolerance.

The importance of secretion

A polarized secretion system is fundamental to fungal cell wall biosynthesis, filamentous growth and interactions with the environment (Malavazi *et al.*, 2014). Protein maturation and transport commences in the endoplasmic reticulum (ER). Adverse environmental conditions that impact upon proper protein folding, such as high temperature, oxidative stress and hypoxia induce the accumulation of misfolded proteins in the ER lumen causing stress (Richie *et al.*, 2009). Additionally, an increased demand on the secretion system, due to nutritional imbalances or the exposure to cell wall damaging agents, can also causes ER stress. In turn, the unfolded protein response (UPR) maintains ER functionality by increasing secretion potential and limiting protein translation (Malavazi *et al.*, 2014). In *Saccharomyces cerevisiae*, *Aspergillus nidulans* and *Trichoderma reesei* ER stress also induces CpcA transcription and the cross-pathway control (CPC) mechanism for regulating amino acid biosynthesis (Arvas *et al.*, 2006), suggesting that CPC may also overlap with UPR in *A. fumigatus*, as is the case in other fungi (Fig. 2). Disruption of the ER stress response in *A. fumigatus*, via the creation of Δ*hacA*, Δ*ireA*, and Δ*cpcA* mutants, all resulted in attenuated virulence (Krappmann *et al.* 2004; Richie *et al.*, 2009; Feng *et al.*, 2011). In addition, the Δ*hacA* and Δ*ireA* mutants showed increased sensitivity to cell wall destabilising agents, echinocandin antifungals, plus reduced tolerance of hypoxia and heat shock (Richie *et al.*, 2009; Feng *et al.*, 2011). Conversely, the $PkcA^{G579R}$ mutant showed increased sensitivity to ER stress inducers which induce MpkA phosphorylation (Rocha *et al.*, 2015). Therefore, UPR maintains ER functionality and protein secretion, governing hypoxia adaptations, iron and nutrient acquisition from the host, tolerance to antifungal compounds, cell wall homeostasis and virulence. Consequently, the UPR has been described as a virulence hub in *A. fumigatus* (Krishman and Askew, 2014) due to the broad ranging impact on numerous stress responses fundamental to host infection.

The development of combinational therapeutics to target fungal stress responses

New therapies to combat IA are required due to the high mortality rates and increasing incidence of IA. Combinational therapies utilise multiple drugs with differing pharmacological targets and complementary mode of action. The benefit of combinational antifungal therapies has been demonstrated in combating other human diseases such as HIV, tuberculosis and cryptococcal meningitis, while the success of such an approach at treating IA, *in vitro* and *in vivo*, appears varied (Steinbach *et al.*, 2003). The ideal antifungal drug has broad spectrum activity and would target a fungal-specific protein, to the extent that its inhibition is not toxic to the patient, while being essential to fungal viability or functions as a virulence determinant. Therefore, the aforementioned highly conserved fungal stress response pathways may fit these criteria and represent potential targets for the development of novel therapeutics.

The absence of a cell wall in humans and its essentiality to fungi make the cell wall biosynthetic machinery promising targets for antifungals. In fact, fungal cell wall targeting echinocandins are now used to treat IA. However, due to a paradoxical effect where echinocandins become less effective at high concentrations and the rise of resistance strains, the efficacy of this mono-therapy has decreased. The simultaneous inhibition of fungal cell wall biosynthesis and the disruption of the fungal cell membranes through the combinational administration of echinocandins with Amphotericin B or azoles appear to have a synergistic effect (Steinbach *et al.*, 2003). Additional compounds targeting fungal cell wall biosynthesis remain an attractive option for future development. The calcium signalling calcineurin pathway is fundamental for fungal growth and virulence, while performing a role in the maintenance of cell wall integrity and the echinocandin-in-

duced stress response. In accordance, immuno-suppressive calcineurin inhibitors, cyclosporine A and FK506, are active against fungi (Juvvadi *et al*., 2014), and show the potential to be developed into a synergistic combinational strategy. Hsp90 interacts with calcineurin, regulates stress-signalling and promotes genetic variation. Inhibition of Hsp90 with geldanamycin blocks the rapid evolution of antifungal resistance and enhances the echinocandin activity against resistant infections. Combinational therapies which inhibit Hsp90 and cell wall biosynthesis, via the simultaneous administration of geldanamycin and an echinocandin, rescued *Galleria mellonella* larvae infected with *A. fumigatus* (Cowen *et al*., 2009), while the impact of combinational therapy on IA in a mammalian host is unknown. Therefore, strategies to target fungal cell wall stress responses may mitigate the rise of echinocandin resistance and increase the efficacy of dual azole-echinocandin therapies.

Withstanding the host-derived or antifungal-induced oxidative stress is key to IA and antifungal resistance. Knowledge of the *A. fumigatus* oxidative stress response may present practical solutions to combat disease. In accordance, immunomodulatory therapy, which uses cytokines to increase the oxidative burst, has been applied with some success in chronic granulomatous disease patients (Chauhan *et al*., 2006). Natural products can also be used to target cellular stress responses in fungal pathogens and can serve as alternatives/additives to commercial antifungal agents. Inhibitors of the mitochondrial respiratory chain complex III with berberine or veratraldehyde enhanced activity of strobilurin antifungals against *A. fumigatus* and prevented the Δ*sakA* mutant from escaping fludioxonil toxicity (Kim *et al*., 2007). Similarly, the combination of thymol, a natural phenolic compound, with azoles or Amphotericin B, had an additive effect and lowered the required dosage of the antifungal, while genetic analyses implied that thymol impacted upon the SakA oxidative stress response pathway (Kim *et al*., 2010). Therefore, the oxidative stress response can be targeted by phenolics, which in combination with antifungals, can effectively suppress the fungal growth. Such natural compounds could serve as useful additives to combinational therapies.

The efficacy of Amphotericin B and echinocandins is enhanced by hypoxia, while varying results have been reported for azoles (Wezensky *et al*., 2011). However, drug access to the fungal biomass may be affected by the reduced perfusion of the hypoxic, necrotic, lesion. In addition, hypoxia may influence the regulation of drug targets, such as the hypoxia-mediated transcriptional induction of the 14-demethylases encoding gene e*rg11A/cyp51A*, which is involved in sterol biosynthesis and is the target of azole fungicides, potentially influencing the synergistic impact of the two stress conditions (Wezensky *et al*., 2011). Therefore, a better definition of the interconnected response to stresses experienced by the fungal pathogen within the host and antifungal treatment may provide new routes to inhibit *A. fumigatus* coping mechanisms, dually reducing the viability of the pathogen within the host environment and increasing the efficacy of the antifungal drug at combating infection.

The aforementioned examples demonstrate how breakthroughs in the understanding of *A. fumigatus* cell signalling and stress response mechanisms can provide viable avenues for the development of novel combinational antifungal therapies. The increased frequency of large-scale forward and reverse genetic studies of fungal pathogens has facilitated, and will continue to assist, in the identification of fungal targets involved in stress responses essential for virulence and antifungal resistance. The subsequent challenge will be the design strategies, such as interfering antibodies or small molecules, that impact specifically on the fungal targets without having cross reactivity in the patient, which may possess orthologous proteins, as is the case with Hsp90. However, such an integrated combinational approach will prove essential in the development of broad range antifungal strategies with high efficacy and low toxicity, which are resilient to the rapid rise of resistance mechanisms.

Conflicting interests

The authors have no conflicting interests.

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