UNIVERSIDADE DE SÃO PAULO FACULDADE DE MEDICINA DE
RIBEIRÃO PRETO

Lilian Pereira Silva

**Caracterização dos fatores de transcrição que têm o domínio
zíper de leucina básica (bZIP) ATF1 em *Aspergillus fumigatus***

Ribeirão Preto

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Tese de Doutorado apresentada à Faculdade de
Medicina de Ribeirão Preto Universidade de São
Paulo para obtenção do título de Doutora em
Ciências.

Área de Concentração: Bioquímica

Orientador: Prof. Dr. Gustavo Henrique Goldman

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“O sucesso nasce do querer, da determinação e persistência em se chegar a um objetivo. Mesmo não atingindo o alvo, quem busca e vence obstáculos, no mínimo fará coisas admiráveis.”

-José de Alencar

RESUMO

Caracterização dos fatores de transcrição que têm o domínio zíper de leucina básica (bZIP) ATF1 em *Aspergillus fumigatus*.

O fungo *Aspergillus fumigatus* é um importante patógeno oportunista e alérgeno de mamíferos, causando doenças tais como a aspergilose pulmonar invasiva, doença que acomete, principalmente pacientes imunocomprometidos ou sob tratamentos quimioterápicos. *A. fumigatus* possui uma grande capacidade de adaptação frente a diferentes condições de estresse, sendo capaz de modular a sua maquinaria bioquímica de forma rápida e robusta, garantindo assim a sua sobrevivência. A via de glicerol de alta osmolaridade (HOG) está envolvida nesses processos de adaptação. As quinases MpkC e SakA de *A. fumigatus*, ortólogas da *Saccharomyces cerevisiae* Hog1p, constituem o regulador primário da resposta ao estresse hiperosmótico ativados pela via de HOG, um dos sistemas responsáveis pela modulação bioquímica e transcricional frente a diferentes condições de estresse. A resposta transcricional ao estresse osmótico, avaliada por RNAseq, foi comparada entre o tipo selvagem e os mutantes $\Delta mpkC$, $\Delta sakA$ e $\Delta mpkC \Delta sakA$. Os resultados indicam que MpkC e SakA têm funções independentes e colaborativas durante a resposta transcricional ao estresse osmótico transiente. Foram também caracterizados os mutantes nulos de *A. fumigatus* para quatro fatores de transcrição com o domínio do tipo zíper de leucina básica (bZip), durante a adaptação a diferentes condições de estresse. Ao longo do estudo, observou-se que os TFs estão envolvidos na capacidade de *A. fumigatus* se adaptar a diferentes agentes estressores que causam danos à parede celular (tais como Congo Red, CR e Calcofluor White, CFW) e estresse oxidativo (p.ex., peróxido de hidrogênio). Primeiramente, nós investigamos as interações, interações genéticas entre $\Delta atfA$, $\Delta atfB$, $\Delta atfC$ e $\Delta atfD$. Os nossos resultados indicam que existem diversos níveis de interações genéticas entre estes mutantes. Entretanto, as mutações $\Delta atfA$ e $\Delta atfB$ mostram epistasia na maior parte das condições aqui investigadas. Todos os duplos mutantes têm crescimento reduzido na presença de acetato,

glicerol e etanol como fontes únicas de carbono, e afetaram o acúmulo de glicogênio e trealose no micélio.

Além disso, foi demonstrado através de microscopia de fluorescência, que a proteína AtfA:GFP é constitutivamente localizada no núcleo. AtfB, -C e D:GFP foram localizadas constitutivamente apenas 20% no núcleo, aumentando a sua translocação para o núcleo entre 30 and 70 % na presença de condições de estresse. Foi também observado, através de experimentos de co-imunoprecipitação (Co-IP), a interação de AtfA-D com SakA. Também ocorre a formação de heterodímeros entre AtfA-D na presença e ausência de estresses osmótico e de parede celular. Esses dados apontam a importância de AtfA-D e ainda sugerem que esses TFs estão envolvidos não somente na resposta ao estresse, mas também em processos metabólicos de *A. fumigatus*.

Palavras chaves: *Aspergillus fumigatus*, Via HOG, estresse osmótico, virulência, AtfA, AtfB, AtfC, AtfD

ABSTRACT

Characterization of transcription factors containing the basic leucine zipper (bZIP) ATF1 domain in *Aspergillus fumigatus*

The fungus *Aspergillus fumigatus* is an important opportunistic pathogen and allergen in mammals, causing diseases such as invasive aspergillosis, which mainly affects immunocompromised patients or those undergoing chemotherapeutic treatment. *A. fumigatus* has a great adaptation ability when faced with different stressful conditions. The fungus is able to modulate its biochemical machinery in a fast and robust way, thus guaranteeing its survival. The high osmolarity glycerol (HOG) pathway is involved in these adaptation processes. The MpkC and SakA kinases of *A. fumigatus*, orthologous of *Saccharomyces cerevisiae* Hog1p, constitute the primary regulator of the response to hyperosmotic stress that is activated by the HOG pathway, one of the pathways responsible for biochemical and transcriptional modulation under stress. After performing RNAseq under osmotic stress, the transcriptional responses of wild type *A. fumigatus* and the strains $\Delta mpkC$, $\Delta sakA$ and $\Delta mpkC \Delta sakA$ were compared. The results strongly indicate that MpkC and SakA have independent and collaborative roles during the transcriptional response to transient osmotic stress. In addition, null mutants for four transcription factors containing the basic leucine zipper (bZIP) domain were identified and characterized. The involvement of the bZIP transcription factors (TF) AtfA, -B, -C and -D during adaptation to different stressful conditions was assessed. These TFs are involved in the ability of *A. fumigatus* to adapt to different stressful agents, such as congo red (CR) and calcofluor white (CFW), which damage the cell wall, and they are also involved in the response to oxidative stress caused by hydrogen peroxide.

First, we investigate the interactions, genetic interactions between $\Delta atfA$, $\Delta atfB$, $\Delta atfC$ and $\Delta atfD$. Our results indicate that there are several levels of genetic interactions between these mutants. However, the $\Delta atfA$ and $\Delta atfB$ mutations show epistasis in most of the conditions investigated here. All double mutants

have reduced growth in the presence of acetate, glycerol and ethanol as the sole sources of carbon, and have affected the accumulation of glycogen and trehalose in the mycelium.

In addition, it was demonstrated through fluorescence microscopy that the AtfA:GFP protein is constitutively located in the nucleus. AtfB, -C and D:GFP were constitutively located only 20% in the nucleus, increasing their translocation to the nucleus between 30 and 70% in the presence of stress conditions. It was also observed, through co-immunoprecipitation (Co-IP) experiments, the interaction of AtfA-D with SakA. The formation of heterodimers between AtfA-D also occurs in the presence and absence of osmotic and cell wall stresses. These data point to the importance of AtfA-D and also suggest that these TFs are involved not only in the response to stress, but also in metabolic processes of *A. fumigatus*.

Key words: *Aspergillus fumigatus*, Via HOG, osmotic stress, virulence, AtfA, AtfB, AtfC, AtfD

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1.0 Introdução

INTRODUÇÃO

1.1. Os fungos filamentosos

O Reino fungi é um clado altamente diversificado de eucariotos, incluindo leveduras, fungos filamentosos, cogumelos, entre outros, encontrados em praticamente todos os ambientes, devido as suas habilidades de crescer em praticamente qualquer substrato e sob condições adversas (MAIS et al., 2010; EGBUTA et al., 2017; RICHARDS et al., 2017; HIGUCHI e STEINBERG, 2015).

Os fungos desempenham papéis importantes na reciclagem de nutrientes, e podem agir como predadores, patógenos e parasitas de uma infinidade de outros organismos (NAJARO-ORTIZ e GABALDÓN, 2019). São capazes de secretar e sintetizar uma variedade de produtos naturais como metabolitos primários e secundários, e enzimas que promovem a degradação e a decomposição de produtos, permitindo sua absorção ou assimilação (HIGUCHI e STEINBERG, 2015). Os fungos filamentosos estão diretamente envolvidos na produção de metabólitos primários e secundários. Devido a essas características, os estudos envolvendo fungos filamentosos são de grande importância para a compreensão dos princípios fundamentais da biologia das células eucarióticas (JIANG et al., 2013).

O gênero *Aspergillus* é composto por mais de 350 espécies, exibindo uma grande variação morfológica e estilo de vida como, por exemplo, habitat e patogenicidade, além de diferentes propriedades metabólicas (FRISVAD; LARSEN, 2015). O fungo *Aspergillus nidulans* é provavelmente o mais conhecido, representando um organismo modelo para extensivos estudos genéticos, metabólicos, de desenvolvimento e de regulação celular (BRANDL; ANDERSEN, 2017). Além disso, a manipulação genética de *A. nidulans*, por cruzamento sexual, tem sido crucial para designar seu papel como organismo modelo genético (BRANDL; ANDERSEN, 2017).

Alguns outros fungos do gênero são também conhecidos por serem de ampla ocorrência e causarem, por exemplo, mofos em produtos alimentícios. É o caso de *A. niger*, um grande produtor de enzimas capazes de degradar a

biomassa vegetal (BENOIT et al., 2015). Embora esse arsenal de enzimas degradadoras seja responsável por permitir o crescimento de *A. niger* em alimentos, tais enzimas são interessantes para a biotecnologia. As enzimas glicose oxidases utilizadas para a quantificação do açúcar no sangue e a α -amilase, amplamente utilizada no processamento de amido na indústria alimentícia, são conhecidos exemplos (DUBEY et al., 2017). Outro membro do gênero é *A. oryzae*, que se destaca pela capacidade de secretar ácidos orgânicos, principalmente ácido cítrico, em quantidades muito elevadas. Ambos *A. niger* e *A. oryzae* são reconhecidos como organismos seguros (GRAS – *Generally Recognised As Safe*) para a produção de enzimas utilizadas nas indústrias biotecnológica e de alimentos (KAPLAN, 1987).

Além dos gêneros relevantes para as indústrias biotecnológicas, existem outras espécies que são conhecidas principalmente por serem patógenos para seres humanos, podendo causar infecções sistêmicas de risco (KNIEMEYER et al., 2011). Ao longo dos anos, de 1970 até a atualidade, diferentes espécies de fungos filamentosos pertencentes a diversos gêneros, vêm sendo citados devido as inúmeras infecções causadas pelos mesmos (Ahmadi et al., 2012). As infecções causadas por fungos filamentosos podem ocorrer em diferentes órgãos e tecidos como olhos, ouvidos, cavidade nasal, unhas, pele, vias respiratórias, vias internas (e. g. órgãos) entre outros (NAJARO-ORTIZ e GABALDÓN, 2019).

Dentre elas, podemos citar *Aspergillus fumigatus*, que ocupa o posto de fungo filamentoso mais prevalente no ar e é o principal causador de aspergilose (KNIEMEYER et al., 2011). Essas infecções são de difícil erradicação e, por isso, são conhecidas como potencialmente ameaçadoras à vida humana (BRANDL; ANDERSEN, 2017).

1.2 O patógeno *A. fumigatus*

O fungo filamentoso *A. fumigatus* pertence à família dos Trichocomaceae, descrito pela primeira vez por Fresenius em 1863, e possui um genoma haploide com 29,4 milhões de pares de bases (BANDRES; SHARMA, 2018). Esses

ascomicetos produzem ascos contendo ascósporos (esporos sexuais) ou conídios (esporos assexuais). Os conídios de *A. fumigatus* possuem aproximadamente 2 a 5 µm e esses conídios podem crescer de forma polar como estruturas tubulares conhecidas como hifas, que são estruturas cilíndricas com uma extremidade característica cuja forma se parece com um hemilipsoide, possuindo múltiplos núcleos num citoplasma único (DAVID M. GEISER, 2009; ROSA R. MOURINO-PEREZ, 2013; RIQUELME et al., 2018).

Existem aproximadamente 60 espécies de *Aspergillus* com importância clínica significativa, sendo a maioria das infecções causadas por *A. fumigatus*, *Aspergillus flavus*, *A. niger* ou *Aspergillus terreus* (YU et al., 2020). As características morfológicas, tais como as capacidades de tolerância ao estresse, de evitar ou tolerar o sistema imune do hospedeiro, de colonizar e/ou danificar os tecidos do hospedeiro, entre outros aspectos, permitem que *A. fumigatus* seja um patógeno bem sucedido (SHANKAR et al., 2018; GLAMPEDAKIS et al., 2020).

Devido ao pequeno tamanho dos conídios, estes podem permanecer suspensos no ar por um longo período de tempo, chegando a uma concentração que varia entre 1 e 100 conídios por m³, podendo atingir a concentração de até 10⁸ conídios por m³ em certos ambientes (WERY, 2014; CADENA et al., 2016). Centenas desses conídios suspensos são inalados por seres humanos, chegando ao trato respiratório (SHANKAR et al., 2018).

As infecções pulmonares causadas por *A. fumigatus*, ocorre pela inalação dos conídios transportados pelo ar, no entanto, de um modo geral, as infecções fúngicas dependem da resposta imune do hospedeiro, ou seja, só há infecção quando há uma falha no sistema de defesa (GAGO et al., 2018). Por outro lado, recentemente foi descoberto que a colonização por *Aspergillus* tem uma associação genética, relacionada à expressão inadequada do fator de transcrição *ZNF77* nos brônquios epiteliais, levando a danos na integridade da parede das células epiteliais e à regulação positiva da matriz extracelular (ECM) que facilitam a adesão dos conídios nas células do hospedeiro (GAGO et al., 2018)

Os conídios representam a principal forma de reprodução de *A. fumigatus*. Os conídios são metabolicamente inativos, no entanto, são capazes de responder a estímulos ambientais, reativando suas maquinarias bioquímicas e genéticas, processo esse chamado de germinação (LATEGÉ e CHAMILOS, 2019). Durante a infecção pulmonar por *A. fumigatus*, a germinação dos conídios é um passo fundamental para o estabelecimento da aspergilose invasiva. A germinação dos conídios pode ser dividida em quatro etapas: (i) a quebra da dormência dos conídios; (ii) o aumento de volume isotrópico; (iii) o estabelecimento da polaridade celular; e (iv) a formação dos tubos germinativos e a manutenção do crescimento polar (BARHOOM; SHARON, 2004).

As espécies de *Aspergillus* causam um amplo espectro de doenças em humanos (VEERDONK et al., 2017). *A. fumigatus* destaca-se dentre os fungos filamentosos por sua predominância no ambiente e pelo seu potencial de causar doença pulmonar obstrutiva crônica (COPD), asma e fibrose pulmonar, entre outras (KNIEMEYER et al., 2011; PRASAD et al., 2016). No mundo, os dados apontam que mais de 6,5 milhões de pessoas apresentam asma grave com sensibilização fúngica (SAFs) e aproximadamente 4,8 milhões de adultos têm aspergilose bronco-pulmonar (ABPA), ambas causadas por *A. fumigatus*, que também é responsável por causar 90% das síndromes de aspergilose pulmonar. Esse patógeno oportunista atinge hospedeiros imunocomprometidos, causando sérias doenças alérgicas e aspergilose invasiva (BARNES et al., 2006; LANG-YONA et al., 2016). No Brasil, estudos apontam que o número de mortes causadas por infecções fúngicas é semelhante aos números de mortes causadas por tuberculose ou malária (GIACOMAZZI et al., 2016).

A aspergilose pulmonar crônica (CPA) é uma infecção fúngica grave, que afeta aproximadamente 3 milhões de pessoas em todo o mundo, sendo um problema de saúde pública significativo, porém negligenciado (BARAC et al., 2019). A CPA possui similaridade com a tuberculose, a histoplasmoses pulmonar e a coccidioidomicose (HAYES e NOVAK-FRAZER, 2016). Além disso, a aspergilose invasiva (IA) é uma das complicações infecciosas graves com um alto índice de morbidade e mortalidade. Os pulmões são os órgãos mais comumente afetados por IA devido à via de infecção ser inalatória. Durante uma

revisão de 139 casos de IA, 79,9% das infecções foram diagnosticadas nos pulmões. Além disso, 12% são cutâneas, 5% são sinusais, 2% são cerebrais e 1,1% é traqueobronquite (BURGOS *et al.*, 2008). Além disso, a infecção pode ser disseminada dos pulmões à parede torácica e ao coração, ou ainda passar do sistema sinusal para o sistema nervoso central (SNC) (BROENEN *et al.*, 2014).

Apesar de alta gravidade, a infecção afeta principalmente pacientes com neutropenia, nos quais se pode encontrar a formação de hifas no tecido pulmonar, chamado de aspergilose pulmonar invasiva (IPA), provocando acidente vascular cerebral e trombose vascular quando ocorre disseminação extrapulmonar (TORO-LEZ CANO *et al.*, 2015). O número de infecções causadas por *A. fumigatus* vem aumentando diariamente, no entanto, a sua patogenicidade ainda é pouco compreendida (MASCHMEYER, HAAS e CORNELLY, 2007; LATGÉ, 2001).

A inalação dos esporos de *A. fumigatus* por indivíduos saudáveis não oferece grandes riscos à saúde, já que esses esporos são eliminados pelas defesas pulmonares, onde os macrófagos alveolares (AM) são a primeira linha de defesa contra os conídios inalados (BANDRES; SHARMA, 2018). Os macrófagos normalmente reagem ao reconhecer os principais componentes da parede celular dos fungos, como o β -1,3D-glucano, secretando mediadores inflamatórios que, em última instância, atraem os neutrófilos para iniciar a imunidade celular (RIQUELME *et al.*, 2018).

Os AM possuem receptores como “*Toll-like*”, dectina-1 e lectina de ligação à manose, que identificam componentes da parede celular fúngica e produzem citocinas para estimular o recrutamento de neutrófilos, sendo este o principal mecanismo de defesa contra as hifas de *A. fumigatus*. No entanto, sabe-se que muitas espécies de *Aspergillus* produzem metabólitos tóxicos (aflatoxinas, micotoxinas, ácido 3-nitropropiónico e ocratoxina A), que inibem a ação dos macrófagos e a fagocitose dos neutrófilos (BANDRES; SHARMA, 2018; KOUSHA *et al.*, 2011; ZHANG *et al.*, 2015). Em pacientes imunocomprometidos, esses conídios alcançam os alvéolos, iniciam o processo de germinação, produzindo hifas, que, por endocitose, invadem o parênquima pulmonar, crescendo dentro das células epiteliais do pulmão (BROENEN *et al.*, 2014).

Na maioria das vezes, a aspergilose é assintomática. Quando sintomática, os sintomas mais comuns são a pneumonia disseminada crônica com expectoração, tuberculose pulmonar, exacerbação de fibrose cística, tosse e falta de ar (KOUSHA *et al.*, 2011; ZHANG *et al.*, 2015). O prognóstico depende do espectro da infecção, sendo a aspergilose broncogênica alérgica a infecção mais leve, e aspergilose invasiva de espectro mais grave. A infecção causada por fungos requer tratamento com antifúngicos como itraconazol, voriconazol, posaconazol, isavuconazol, anfotericina B e micafungina, pertencentes às classes dos azóis e polienos, cujo o alvo é a membrana fúngica (MROCZYNSKA *et al.*, 2020). No entanto, a escolha da terapia antifúngica deve ser minuciosa e levar em consideração a espécie de *Aspergillus*. Em casos de progressão severa e deterioração do estado do paciente, tratamentos com antifúngicos da classe das equinocandinas, cujo o alvo é a parede celular fúngica, devem ser considerados. Portanto, o diagnóstico precoce é importante para que o tratamento seja mais eficaz (BANDRES; SHARMA, 2018).

Existem muitos desafios a serem superados quando falamos em infecções fúngicas humanas, principalmente devido aos fungos eucarióticos compartilharem estruturas celulares e vias metabólicas semelhantes às daquelas dos seres humanos (BROWN *et al.*, 2012). As opções de drogas antifúngicas são limitadas e estão repletas de efeitos colaterais aos seres humanos (BROWN *et al.*, 2012). Além disso, recentemente, múltiplos casos de resistências às drogas antifúngicas têm sido relatados. Assim, há uma grande necessidade de novas drogas antifúngicas com efeitos colaterais reduzidos para os pacientes (BRANCH *et al.*, 2016). Uma estratégia para descobrir novos alvos terapêuticos para drogas com potenciais antifúngicos é direcionar estudos para as vias metabólicas fúngicas, avaliando a maquinaria bioquímica e os mecanismos pelos quais ocorrem as interações fungo-hospedeiro (THAMMAHONG *et al.*, 2017).

Ao longo dos últimos anos, vem sendo demonstrado que a virulência de *A. fumigatus* é multifatorial, e algumas características biológicas contribuem para a virulência (LATGÉ e CHAMILOS, 2019). Por exemplo, a capacidade do fungo de se adaptar às tensões, em especial a adaptação ao estresse oxidativo. A

adaptação a esse tipo de estresse é fundamental para a patogênese, já que o sistema imune do hospedeiro causa estresse oxidativo nos esporos inalados, sendo esse o principal mecanismo de defesa LATGÉ e CHAMILOS, 2019).

1.3 A via de sinalização das proteínas quinases ativadas por mitógeno (MAPK)

Entre as várias vias de sinalização presentes em um sistema eucariótico, a via da proteína quinase ativada por mitógeno (MAPK), é uma das vias de sinalização mais importantes (MOHANTA et al., 2016). Os fungos possuem diferentes proteínas quinases, organizadas sob a forma de um sistema de cascatas de quinases (MAPK), que são induzidas e capazes de responder a uma ampla variedade de agentes estressores (DOHLMAN e THORNER, 2001; MOHANTA et al., 2016). As vias de MAPK são mais bem compreendidas em leveduras, mas sabe-se que em fungos filamentosos, essas vias estão envolvidas em diferentes processos fisiológicos incluindo o ciclo celular, a morfogênese, a esporulação, a integridade da parede celular, a patogênese, a tolerância à radiação ultravioleta, a tolerância ao choque térmico, a sinalização das células e a interação fungo-fungo (FU et al., 2016).

As cascatas de MAPK constituem um dos mecanismos mais importantes e evolutivamente conservados em todos os organismos eucarióticos. São estimuladas através de cascatas de proteína quinase, conectadas a sensores ou receptores na superfície celular, culminando em dupla fosforilação em resíduos de tirosina (Tyr) e treonina (Thr) (LEE; LIU; LEVIN, 2018). Essas cascatas de quinases consiste em três proteínas quinases que atuam em série: quando uma cascata de MAPK é ativada, a MAP quinase quinase quinase (MAPKKK) fosforila a MAP quinase quinase (MAPKK), que, por sua vez, fosforila a MAP quinase (MAPK). A ativação sequencial das quinases resulta numa amplificação do sinal de iniciação, resultando na modulação de fatores de transcrição, que finalmente regulam a expressão gênica em resposta ao fator de ambiental (GUSTIN et al., 1998; (LEE; LIU; LEVIN, 2018; GONZALES-RUBIO et al., 2019).

A. fumigatus possui quatro MAPKs importantes com funções relacionadas a sua capacidade de adaptação e tolerância: (a) MpkA, regulador central da via de integridade de parede celular (CWI) (ROCHA et al., 2020). (b) MpkB, regula a resposta a feromônios, e ainda a conidiação e a produção de melanina diidroxinaftaleno (DHN) através da via de crescimento filamentososo (MANFIOLLI et al., 2019). (c) SakA e (d) MpkC, que desempenham papéis importantes na tolerância a caspofungina, alta osmolaridade e estresse oxidativo, através da ativação da via HOG (GARRIDO-BAZÁN et al., 2019).

Os processos de transdução de sinal pelas MAP quinases, conforme mencionado anteriormente, se iniciam após a detecção de estímulos ambientais, o que ocorre através de receptores e proteínas ancoradas à membrana celular, que transduzem o sinal externo para proteínas intracelulares (GONZALES-RUBIO et al., 2019), por exemplo, os sistemas de transdução de sinal de dois componentes (TCS), as tirosinas quinases receptoras (RTKs) ou estruturas de multiproteínas que estão conectadas a proteínas G heterodiméricas ou monoméricas acopladas a receptores (ALVARO e THOMER, 2016). Na Figura 1 está representado um esquema geral da via MAPK (MARTÍNEZ-SOTO e RUIZ-HERRERA, 2016).

Figura 1- Esquema representativo do mecanismo de sinalização das vias MAPK em fungos. Dentre os possíveis sinais detectados e respostas aos estressores estão: Transdução de sinal de dois componentes (TCS), receptores acoplados a proteína G, tirosina quinases receptoras (RTKs) e a interação de MAPK com outras vias de sinalização, por exemplo, a via de proteína quinase A (PKA) dependente de cAMP. São utilizadas as abreviaturas seguintes: MAPKKK, quinase quinase; MAPKK, quinase; MAPK; TF fatores de transcrição; HK, histidina quinase; RR, regulador de resposta; HPT, domínio de fosfotransferência. (MARTÍNEZ-SOTO e RUIZ-HERRERA, 2016).

1.3.1 Via de glicerol de alta osmolaridade (HOG)

Um dos modelos de MAPK melhor elucidado, é a via de glicerol de alta osmolaridade (HOG) no modelo *Saccharomyces cerevisiae*. Esta via é responsável por responder a diferentes estímulos, dentre eles, o crescimento filamentoso, o estresse osmótico, a modulação de diferentes características na virulência, incluindo crescimento invasivo, a formação de biofilme e ainda a tolerância a alguns antifúngicos (JUN-JIAO et al., 2019). Várias proteínas conservadas que abrangem a membrana plasmática possuem função de biosensores de processos celulares, para ativar a cascata de MAPK a jusante, essas proteínas incluem o ramo Sho1, composto por uma mucina Msb2, Hkr1, Opy2 e o ramo Sln1 histidina quinase (KOU e NAQVI, 2016).

Em *S. cerevisiae*, a MAPK Hog1p é ativada por estresse hiperosmótico, através da via de HOG que orchestra uma série de mudanças osmoadaptativas (TATEBAYASHI et al., 2020). A via HOG1 é ativada pela fosforilação e translocação nuclear de Hog1p, culminando na regulação da tradução de

proteínas, expressão ou repressão de vários genes, além dos ajustes para a progressão do ciclo celular (ZHAO *et al.*, 2007). Em mais detalhes, no modelo *S. cerevisiae*, a via HOG possui duas diferentes ramificações *upstream*, o ramo SLN1 e o ramo SHO1, uma cascata central de MAPK e vários outros reguladores, que são os alvos da via (MA e LI, 2013; JIMÉMEZ-GUTIÉRREZ *et al.*, 2020). O aumento da osmolaridade externa é captado pelas proteínas “osmosensores” Sln1p e Sho1p, que conduzem o sinal de forma independente para a fosforilação de MAPK (MAPKK). Sln1p por sua vez ativa duas quinases Ssk2p e Ssk22p (MAPK, MAPKKK) que então ativam Pbs2p. Uma vez fosforilado e ativado, o Pbs2p fosforila e ativa Hog1p^{p38^{MAPK}}, que é translocada rapidamente do citoplasma para o núcleo, assim regulando processos diferentes, entre eles o acúmulo de glicerol, compensando assim o aumento da osmolaridade externa (NADAL, AMMERER e POSAS, 2011; ZI *et al.*, 2010; TABAYASHI *et al.*, 2020). Além disso, Sho1p também pode desencadear o sinal para a fosforilação de MAPK, possivelmente mediando interações proteína-proteína (PPI) através das proteínas mucina Msb2p e Hrk2p, que levam ao recrutamento de Rho GTPase (Cdc42p), um regulador global de polaridade celular e sinalização para a âncora de membrana Opy2p, ativando então Ste20p, um mediador chave da fosforilação de Hog1p que, subsequentemente, ativa Ste11p. Sho1p e o complexo Opy2-Ste50 recrutam Pbs2p, permitindo então que Pbs2p seja fosforilado por Ste11p para detectar o aumento da osmolaridade extracelular (LAM *et al.*, 2015; MANFIOLLI *et al.*, 2019; TAKAYAMA *et al.*, 2019).

Em *C. albicans*, Sho1p, além de estar envolvido na adaptação ao estresse osmótico e oxidativo, também contribui para a adaptação a agentes que interferem na parede celular (FOR *et al.*, 2005; DUBEY *et al.*, 2017). O receptor Sho1p é essencial para a ativação de Cek1p, a MAPK específica da via de crescimento em fungos filamentosos. A deleção de *SHO1* em leveduras aboliu o crescimento das pseudo-hifas, importantes para o processo invasivo (Correia *et al.*, 2020). Além disso, foi sugerido que a proteína transmembrana Opy2p é um osmosensor no ramo Sho1p em *C. albicans* (LIU *et al.*, 2011). Segundo os estudos apresentados por Dios e colaboradores (2012), Opy2p é necessário para desencadear a fosforilação de Cek1p por diferentes estímulos, ativando

assim a via HOG. Nesse mesmo trabalho foi relatado que o mutante *OPY2* apresentou virulência reduzida no modelo *Galleria mellonella*.

Em *A. fumigatus*, a via HOG possui dois parálogos de Hog1p, MpkC e SakA (NASCIMENTO *et al.*, 2016). Muitos componentes das vias de MAPK foram determinados e caracterizados através de cepas mutantes. No trabalho realizado por Nascimento e colaboradores (2016), foi elucidado o envolvimento de SakA e MpkC na resposta ao estresse osmótico, integridade da parede celular, e virulência, além do seu envolvimento na adaptação à caspofungina, e a utilização de diferentes fontes de carbono (ALTWASSER *et al.*, 2015). Os resultados obtidos através dos estudos realizados com a cepa mutante $\Delta sakA$, mostraram que o mutante foi mais sensível quando exposto a drogas que causam estresse osmótico e dano a parede celular, sugerindo SakA contribui para a adaptação do fungo nestas condições. Ainda, a construção de um duplo mutante $\Delta sakA \Delta mpkC$, levou a atenuação da virulência na cepa mutante, após ensaios realizados em modelo animal (NASCIMENTO *et al.*, 2016). As proteínas SakA e MpkC desempenham funções semelhantes, formando complexos proteicos através de sua interação física, e ainda, contribuem em processos regulados pela via CWI, onde SakA interage não apenas com MpkC, mas também com MpkA (MANFIOLLI *et al.*, 2019).

Muitos dados na literatura apontam o envolvimento das MAPK em diferentes processos de adaptação, formando interação e mediando o *cross-talk* entre as diferentes vias de sinalização. Na via de HOG, SakA e MpkC vem sendo amplamente estudados, devido ao seu envolvimento em diferentes processos de adaptação e tolerância de *A. fumigatus*. Essas quinases fazem parte de uma via de sinal osmótico, mas também estão envolvidas na regulação da resposta ao dano de parede celular, estresse oxidativo, resistência a drogas e estabelecimento de infecção do fungo (MANFIOLLI *et al.*, 2019).

1.4 O sistema de fosforilação de histidina quinase de dois componentes

Os sistemas de fosforilação de histidina quinase de dois componentes são predominantes nos eucariotos, contribuindo para a adaptação osmótica e governando a via HOG. O sistema de dois componentes (TCS), é capaz de

detectar estímulos ambientais por sensores de histidina quinase (HKS) e transmitir esses sinais para os reguladores de resposta (HAGIWARA *et al.*, 2013; MA *et al.*, 2013; DAY e QUINN, 2019).

A TCS é composta por híbridos-histidina (hHKs), que possuem uma HK, um domínio contendo os reguladores de resposta (RR) e uma proteína fosfotransferase contendo histidina (HPT) como um fator intermediário. Algumas histidinas quinases estão envolvidas na regulação osmótica. Em *S. cerevisiae*, Sln1p é o único sensor de histidina quinase, enquanto outros fungos podem ter mais de dez (FURUKAWA *et al.*, 2002; BRANSCUM *et al.*, 2019).

Em fungos, existem 11 grupos distintos de HHKs, classificados de acordo com sua estrutura e função. Em *S. cerevisiae*, Sln1p é o único HHK pertencente ao grupo transmembranar VI. As HKs são proteínas chave de sinalização, estando envolvidas na percepção e na transdução de diferentes estímulos ambientais, além de terem importante papel na regulação da morfogênese e da virulência (BAHN *et al.*, 2006). Quando as células não são submetidas aos estresses externos, a proteína ligada à membrana Sln1p se auto-fosforila em um resíduo de histidina de um domínio chamado H-box, e o fosfato é então transferido para um resíduo de aspartato do domínio receptor, que subsequentemente é transferido para um resíduo de histidina em Ypd1p, uma proteína de fosfotransferência (HPT) e então, finalmente para Ssk1p (CHAUHAN *et al.*, 2006; BRASCUM *et al.*, 2019).

A função das várias HKs fúngicas foram estudadas ao longo dos anos, usando abordagens de genética molecular, principalmente através da obtenção de mutantes (HÉRIVAUX *et al.*, 2016). Em *S. cerevisiae*, Sln1p é necessário para manter a via de HOG em um estado inativo. Assim sendo, a deleção de *SLN1* é letal devido à superativação da via HOG (OTA; VARSHAVSKY, 1993; DUNAYEVICH *et al.*, 2019). Em *C. albicans*, a deleção de *SLN1* não tem grande impacto durante o processo de adaptação ao estresse osmótico. No entanto, a deleção impede a formação de hifas e atenua a virulência do patógeno (NAGAHASHI *et al.*, 1998). Em *A. nidulans*, TcsB, o ortólogo de Sln1p, parece desempenhar um papel menos importante porque a deleção de *tcsB* não impactou o desenvolvimento de hifas ou a morfologia durante o estresse

osmótico, indicando que *tcsB* não é essencial em *A. nidulans* (FURUKAWA et al., 2002). O TcsB de *A. fumigatus* possui uma identidade de aproximadamente 55% com o TcsB de *A. nidulans*, indicando que aquele possui o típico sensor de histidina quinase, com domínios reguladores de resposta e dois domínios transmembranares putativos no N-terminal (DU et al., 2006). Outras funções importantes também estão relacionadas a HKs fúngicas, como, por exemplo, a produção de melanina, a adaptação à hipóxia, regulação do metabolismo secundário e formação de biofilme (HÉRIVAUX et al., 2016).

No modelo *S. cerevisiae*, a fosforilação da histidina quinase Sln1p regula a resposta mediada por Ssk1p citoplasmática, importante para a desaceleração da atividade da via da MAPK HOG1 sob condições osmóticas normais. (FURUKAWA et al., 2002; FASSLER and WEST, 2010; DUNAYEVICH et al., 2019). Em tais condições, a histidina quinase Sln1p fosforila a proteína fosfotransferase Ypd1p, que medeia a ativação das vias Ssk1p e Skn2p, esta última importante na resposta à agentes perturbadores de parede celular. Por outro lado, o aumento da osmolaridade regula negativamente a atividade de histidina quinase Sln1p, o que, por sua vez, resulta na ativação da cascata da MAPK HOG1p, resultando na ativação de genes responsáveis pela adaptação de estresse osmótico (FURUKAWA et al., 2002; DUNAYEVICH et al., 2019). Podemos observar na Figura 2A que a via HOG possui múltiplas rotas entre a entrada (alta osmolaridade externa) e a saída (respostas dependentes de HOG1). Essas rotas são denominadas ramificação *SLN1* e ramificação *SHO1*. A histidina quinase Sln1p, transmite o sinal através de um mecanismo de fosforilação de dois componentes para as MAPKKs, que eventualmente ativam Hog1p MAPK. Além disso, podemos observar que a via Hog1p também pode ser ativada através do *MSB2*, consecutivamente na cascata Hog1p MAPK, Ste20p, Ste11p e Pbs2p, que são recrutadas do citoplasma para a membrana plasmática pelas proteínas TM, Msb2p, Opy2p e Sho1p, respectivamente. (YAMAMOTO et al., 2016; UDOM et al., 2019).

Figura 2 - Modelo esquemático da via HOG em *S. cerevisiae*.

(UDOM et al., 2019)

No modelo *S. cerevisiae*, a HKs de três componentes Sln1p-Ypd1p-Ssk1p é responsável por manter a via de HOG inativada sob condições normais. A desfosforilação de Ssk1p depende exclusivamente de Ypd1 e que este depende de Sln1p (DEXTER et al., 2015). Segundo Fassler e West (2010), a deleção ou mutação em qualquer um dos resíduos de fosforilação em Sln1p, Ypd1p, ou Ssk1p leva à inviabilidade das células, reprimindo ou inativando componentes de hog1p da via MAPK HOG. Essas informações levaram à conclusão atual de que a via SLN1-YPD1-SSK1 é importante na regulação negativa da atividade da via HOG1 na ausência de alta osmolaridade. A atividade da via *SLN1* é diminuída em condições osmóticas elevadas, sendo que essas condições conduzem à acumulação de componentes de Sln1p desfosforilado. Assim, a não fosforilação de Ssk1p, que ativa as quinases Ssk2p e Ssk22p (MAPKKKs) da via

HOG1p da cascata de sinalização, faz com que haja alterações na expressão de genes através da ativação de fatores de transcrição que são responsáveis pela transdução dos genes envolvidos na resposta ao estresse osmótico, incluindo aqueles envolvidos na biossíntese do glicerol (FASSLER e WEST, 2010).

Outros modelos fúngicos, tais como *Neurospora crassa*, também possuem TCS que estão relacionadas a funções tais como morfologia e conidiogênese, desenvolvimento sexuado, resposta ao estresse oxidativo, a adaptação osmótica e a sensibilidade aos fungicidas (HAGIWARA *et al.*, 2013). Assim sendo, é importante compreender o sistema HOG MAPK e suas extensões, incluindo a interação entre as histidinas quinase.

1.5 O fator de transcrição ATF1, um membro da família de reguladores de transcrição do tipo “basic leucine zipper” (bZIP)

As vias de sinalização de diferentes fungos e leveduras, como *Schizosaccharomyces pombe*, *S. cerevisiae*, *A. nidulans* e *A. fumigatus* induzem fatores de transcrição, que por sua vez, ativam a expressão de diferentes genes alvos (LINZ *et al.*, 2013). Estima-se que existam mais de 90 tipos de fatores de transcrição (TF) em eucariotos, sendo que as principais classes estruturais dos TF sejam, “Zinc Finger” (C_2H_2 e C_4 e agrupamentos de zinco Zn_2 Cys₆ / C₆); “helix-turn-helix” (HTH) e estruturas “ β -scaffold” com domínios básicos zíper de leucina (basic leucine zipper/bZIP) (CARRILLO *et al.*, 2017; WEIRAUCH e HUGHES, 2011).

Os TF desempenham papéis cruciais nas redes regulatórias, são eles que regulam a expressão dos genes alvos, ligando-se a locais específicos (regiões promotoras) no DNA. Os TF bZip, é uma categoria chave nesse processo, exclusivos em eucariotos, esta família de genes desempenha papéis importantes nos processos de desenvolvimento e em várias respostas ao estresse (XU *et al.*, 2020). A característica típica do TF bZip é o domínio bZip, que consiste em aproximadamente 70 aminoácidos, abrigando duas partes funcionais distintas, uma região básica altamente conservada e uma região variável de leucina (LINZ *et al.*, 2013). A região variável (Zip) de leucina é composta por repetições de leucina e outros aminoácidos hidrofóbicos, e exatamente nove resíduos de

aminoácidos em direção ao C-terminal, que favorece a homo e heterodimerização das proteínas bZip (VINSON et al., 1989).

No genoma do *Homo sapiens*, a ativação do TF bZip ATF2 desempenha um papel crítico na proliferação e desenvolvimento do ciclo celular, resposta ao estresse, bem como, resposta ao dano no DNA (INOUE et al., 2019). Além disso, Watson e colaboradores (2017) demonstraram que, em mamíferos, ATF2 é ativado após as células sofrerem tensões desencadeada por radiação UV, hipóxia ou citocinas inflamatórias, atuando na regulação de uma variedade de genes, que estão envolvidos na proliferação, transformação, reparo, inflamação e na apoptose celular, ainda, ATF2 pode ser ativado por várias proteínas quinases, que tem sido implicadas em desempenhar papéis importantes na manutenção e regulação imunológica (INOUE et al., 2019; MEIJER et al., 2020). Além disso, estudos também mostram que os TFs podem regular vários genes associados à resposta imune durante o processo de infecção, tais como as citocinas, TNF α , IL-1 β , IL-6, IL-8, IL-12, entre outros (TISUKADA et al., 2011).

Em leveduras, os TFs medeiam respostas transcricionais precisas à diversas condições ambientais, favorecendo à adaptação e os tornando reguladores mestres no desenvolvimento e no funcionamento celular (HERDEGEN e LEAH, 1998). Em *S. cerevisiae*, o primeiro TF bZip encontrado e caracterizado foi o YAP1 (proteína ativadora de levedura), então, o subconjunto YAP1p-YAP8p demonstraram estar envolvidos na mediação ao desenvolvimento, resposta ao estresse, sexualidade, metabolismo secundário e patogenicidade (MOYE-ROWLEY et al., 1989; WANG et al., 2020).

Os TF bZips pertencentes à família CREB/ATF, são altamente conservados entre as leveduras e fungos filamentosos. Os bZips são controlados não somente pelas MAPKs, mas também pela atividade de cAMP-PKA (proteína quinase A) (STEVEN et al., 2020). Em *S. pombe*, Atf1 é um membro da família bZIP, TF responsável por ativar genes envolvidos na resposta ao estresse, ativado através da cascata de fosforilação de MAPK (MASSON et al., 1993). Após sofrer tensões oxidativas, as quinases Sty1 / Spc1 MAP, são ativadas por fosforilação em resposta ao estresse, desencadeando uma ampla mudança na regulação da expressão genica a nível transcricional (CHEN et al., 2002; SÁNCHEZ-MIR et

al., 2020). Sob estresse, Sty1 é fosforilado, acumulando-se no núcleo para promover a ativação ou a repressão transcricional dos genes, essas mudanças transcricionais são totalmente ou parcialmente dependentes do TF Atf1. Em resposta ao H₂O₂, Chen e colaboradores (2003), demonstraram que Sty1-Atf1 regulam positiva ou negativamente mais de 500 genes em *S. pombe*. Ainda, a ativação de Sty1-Atf1 tem papéis importantes na resposta a “starvation” de nutrientes e ainda à privação de glicose, Atf1 regula transcionalmente genes codificadores da frutose-1,6-bifosfato (*fbp1*), enzima chave na via glucogênica (HOFFMAN e WINSTON, 1991; CHEN et al., 2018; SÁNCHEZ-MIR et al., 2020)

Em *A. nidulans*, o TF bZIP AtfA ortólogo ao Atf1 de *S. pombe* e ATF2 de mamíferos, foi caracterizado como um regulador da tolerância ao estresse conidial (HAGIWARA et al., 2008; Linz et al., 2013). Além disso, AtfA regula vários processos em hifas vegetativas de fungos filamentosos, contribuindo para a tolerância ao estresse e ou alteração no metabolismo secundário em *A. nidulans*, assim como em *Fusarium oxysporum*, *Botrytis cinerea*, *Magnaporthe oryzae* e *Claviceps purpurea* (LARA-ROJAS et al., 2011; NGUYEN et al., 2013; QI et al., 2013; OROSZ et al., 2017).

Em *A. nidulans*, o TF AtfA é responsável por regular a transcrição e a expressão dos genes da catalase B (*catB*) e da catalase A (*catA*) envolvidos na resposta ao estresse oxidativo (LARA-ROJAS et al., 2011; HAGIWARA et al., 2008). Após o fungo perceber o estresse oxidativo, as quinases HogA^{hog1p/SakA} e MpkC são ativadas por fosforilação, que por sua vez ativam os TFs (LARA-ROJAS et al., 2011; GARRIDO-BRAZÁN et al., 2018). As histidinas quinases TcsA/TcsB atuam como osmosensores, captam e transmitem os sinais de estresse oxidativo e osmótico para a cascata HogA^{/SakA} MPKC através de YpdAp^{/SskA} em *A. nidulans* (GARRIDO-BRAZÁN et al., 2018). O mesmo processo é visto para *S. cerevisiae*, sendo que a histidina quinase Sln1p transmite sinais de estresse osmótico para a cascata Hog1p^{HogA/SakA} SAPKp através de Ypd1p e Ssk1p, que por sua vez induz fatores de transcrição como Atf1p^{/AtfA} para a expressão de genes alvo (LINZ et al., 2013).

No patógeno de plantas *B. cinerea*, BcAtf1 homólogo ao AtfA de *A. nidulans*, é induzido após a exposição ao H₂O₂, controlando a expressão de *catB*,

por outro lado, BcAtf1^{/AtfA} não está envolvido diretamente na tolerância de *B. cinerea* ao estresse osmótico. Foi relatado que a deleção do gene *bcatf1*, levou ao acúmulo (2 à 10 vezes mais) de metabolitos secundários, mostrando que BcAtf1 funciona como repressor de genes envolvidos na biossíntese de metabólito secundários, já que o acúmulo de fitotoxinas está correlacionado com a regulação positiva da expressão dos genes envolvidos na biossíntese de toxinas (TEMME et al., 2012; HONG et al., 2013). Além disso, em outros fungos patogênicos de plantas, ortólogos ao AtfA^{/Atf1p} também demonstraram estar envolvidos em diversos processos biológicos: (i) Moatf1, na manutenção do crescimento vegetativo em *Magnaporthe oryzae*; (ii) FgAtf1 na regulação sexual (FgAtf1) em *F. graminearum* e BcAtf1 na regulação assexual em *B. cinerea*. (iii) tolerância e defesa ao estresse oxidativo, osmótico e de integridade da parede celular, CPTF1 em *Claviceps purpurea*, BcAtf1 em *B. cinerea* e (iv) virulência, CPTF1 em *C. purpurea* e Moatf1 em *M. oryzae* (NATHUES et al., 2004; GUO et al., 2010; TEMME et al., 2012; NGUYEN et al., 2013; QI et al., 2013; SZABÓ et al., 2020).

Outro TF pertencente à família dos bZip é o AtfB, ortólogo ao AtfA (OROSZ et al., 2017). Em *Aspergillus parasiticus*, foi relatado que AtfB impacta diretamente na síntese de aflatoxina, os promotores dos genes biossintéticos de aflatoxinas, tais como *nor-1*, *faz-1*, *ver-1* e *omtA*, que contêm sítios CRE, foram relatados como alvo de AtfB (ROZE et al., 2011). Além disso, mutações no sítio de ligação *nor-1* reduz a transcrição após a indução através do cAMP exógeno, indicando que a via cAMP/PKA regula parte da biossíntese da aflatoxina através do AtfB (ROZE et al., 2004). Ainda, o TF AtfB foi considerado um regulador mestre de três redes funcionais, incluindo metabolismo secundário, resposta ao estresse e o desenvolvimento de conídiosporo. Esses processos são importantes, sendo associados a virulência de *A. parasiticus* (ROZE et al., 2011; OROSZ et al., 2017; We et al., 2017). Em *A. oryzae*, a deleção de *atfB* levou a uma menor transcrição de genes que codificam proteínas antioxidantes, como, por exemplo, *catA* (catalase específica de conídios), o que, por sua vez, resultou em conídios sensíveis ao peróxido de hidrogênio (SAKAMOTO et al., 2009).

Hagiwara e colaboradores (2014) investigaram o genoma de *A. fumigatus* na busca de proteínas homólogas ao AtfA^{Atf1p/ATF2}, tendo encontrado quatro proteínas com domínio de ligação ao DNA que são homologas ao AtfA. Essas quatro proteínas, foram denominadas AtfA, AtfB, atfC e AtfD. Em *A. oryzae* e em *A. nidulans*, também podem ser encontrados esses outros três membros de TF, sendo eles AtfB, AtfC e AtfD. No entanto, as sequências de aminoácidos na região bZip de AtfB, AtfC e AtfD são menos conservadas em comparação ao AtfA (HAGIWARA et al., 2014). No entanto, os quatro fatores de transcrição AtfA-D de *A. fumigatus*, possuem aproximadamente 60% de identidade dos aminoácidos em seus domínios (HAGIWARA et al., 2014). Nesse mesmo trabalho, foi realizado a comparação entre os genomas de *A. oryzae*, *A. nidulans* e *A. fumigatus*. Os resultados apontaram que AtfA possui 65,1% de identidade nas três espécies de *Aspergillus*, mostrando que a região bZip é altamente conservada. Além disso, Lara-Rojas e colaboradores (2011) sugeriram que AtfA e AtfB podem interagir formando heterodímeros durante a transcrição de alguns genes; ainda considerando a interação entre AtfA e AtfB na resposta ao estresse oxidativo, é razoável sugerir que AtfA também possa ter uma conexão com o metabolismo secundário. Essa heterodimerização entre os TFs foi relatado anteriormente por Manso e colaboradores (2011) no modelo *S. pombe*, o TF bZip Pcr1 envolvido no desenvolvimento sexual é ativado por cAMP-PKA, e forma heterodímeros com Atf1p, participando na recombinação meiótica, na manutenção da estrutura da heterocromatina e na regulação de genes relacionados à diferenciação sexual.

Os TF estão envolvidos em diferentes mecanismos adaptativos de *A. fumigatus*, no estudo realizado por Hagiwara e colaboradores (2014), foram obtidas cepas mutantes nas quais o gene AtfA foi substituído por um marcador de resistência à higromicina. A linhagem *A. fumigatus* Δ atfA apresentou atraso no crescimento, sensibilidade ao estresse térmico e oxidativo, além de variação no tamanho do conídio quando comparado ao tipo selvagem. Uma análise do transcriptoma também foi realizada durante o desenvolvimento assexuado, comparando o tipo selvagem ao mutante Δ atfA de *A. fumigatus*, os dados obtidos, apontaram que aproximadamente 54 genes são regulados por AtfA durante a conídiação, entre eles foram relatados os genes catalase A (*catA*),

proteína semelhante a deidrina (DprA), proteínas de choque térmico (Scf1), trealose sintase (Ccg9) entre outras.

2.0 Objetivos

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O fungo patogênico *A. fumigatus* possui mecanismos complexos de adaptação, mediados por diferentes vias de sinalização que contribuem, na regulação e na modulação de diferentes genes responsáveis por aumentar a capacidade de adaptação rápida e eficiente do fungo. A busca pela compreensão das vias de sinalização relacionadas a resistência e a capacidade adaptativa tem gerado cada vez mais interesse. A possibilidade de caracterizar interações entre proteínas, fatores de transcrição e até mesmo, *cross-talk* entre as diferentes vias de sinalização, podem contribuir para a descoberta de novos alvos para drogas antifúngicas, possibilitando assim a redução de infecções e contaminações causadas por *A. fumigatus*.

Em *A. fumigatus* a via de HOG vem sendo amplamente estudada, buscando caracterizar e compreender os diferentes mecanismos de interação e ativação das MAPK, assim como, dos fatores de transcrição pelas quinases ativados, incluindo o bZip AtfA, que foi previamente descrito por Hagiwara et al., 2016.

Sendo assim, os principais objetivos do estudo foram:

- (i) A análise do transcriptoma para os mutantes simples $\Delta sakA$, $\Delta mpkC$ e o duplo mutante $\Delta sakA \Delta mpkC$;
- (ii) A caracterização molecular dos genes *atfA*, *atfB*, *atfC* e *AtfD* que codificam quatro fatores de transcrição bZip através da sua deleção e complementação;
- (iii) Caracterizar os duplos mutantes e complementados em diferentes condições estressoras (estresse osmótico, oxidativo e danos a parede celular);
- (iv) Estudar a virulência das cepas mutantes no modelo murino imunossuprimido e no modelo larval *Galleria Mellonella*.
- (v) Construção de cepas expressando fusões GFPs para os quatro fatores de transcrição, para caracterizar a localização celular.
- (vi) Construir cepas SakA:3xHA e AtfA-D:GFP para avaliar a interação entre a quinase e os fatores de transcrição, através de Co-Imunoprecipitação.

- (vii) Avaliar por Co-Imunoprecipitação as interações entre AtfA, -B, -C e -D.

3.0 Apresentação dos Resultados

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Os resultados e discussão aqui apresentados estão divididos em duas partes.

O primeiro capítulo (3.1) apresenta o estudo realizado para investigar a resposta transcricional de *A. fumigatus* ao estresse osmótico, comparando a resposta transcricional do tipo selvagem de *A. fumigatus* ao estresse osmótico com a das cepas mutantes $\Delta mpkC$, $\Delta sakA$ e $\Delta mpkC \Delta sakA$. Essa abordagem revelou fatores de transcrição importantes para a adaptação osmótica dependentes transcionalmente de MpkC e SakA. Análises genéticas subsequentes mostraram a importância do AtfA - D para a adaptação aos estresses osmóticos, oxidativos e da parede celular, além da virulência fúngica. Todo esse estudo levou à publicação do artigo: “Genome-wide transcriptome analysis of *Aspergillus fumigatus* exposed to osmotic stress reveals regulators of osmotic and cell wall stresses that are SakA^{HOG1} and MpkC dependent”, **Silva LP et al.**, 2016, Cell Microbiol.

O capítulo (3.2), é apresentado como uma continuação do trabalho realizado no capítulo 3.1, no qual, buscamos compreender a interação entre os fatores de transcrição AtfA-D durante a adaptação fúngica, frente a diferentes condições estressoras. Ainda, investigamos a hipótese de interação física entre SakA^{HOG1} e os TFs AtfA-D durante a exposição das cepas ao estresse osmótico e de parede celular. Todo esse estudo levou a preparação do artigo: Genetic and protein interactions of the *Aspergillus fumigatus* basic leucine zipper (bZIP) transcription factors AtfA, AtfB, AtfC, and AtfD are important to cope with different stress conditions. Silva LP et al. Submitted to Frontiers in Fungal Biology (Nov/2020).

Capítulo 3.1: Genome-wide transcriptome analysis of *Aspergillus fumigatus* exposed to osmotic stress reveals regulators of osmotic and cell wall stresses that are SakA^{HOG1} and MpkC dependent.

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RESEARCH ARTICLE

Genome-wide transcriptome analysis of *Aspergillus fumigatus* exposed to osmotic stress reveals regulators of osmotic and cell wall stresses that are SakA^{HOG1} and MpkC dependentLilian Pereira Silva¹ | Patrícia Alves de Castro¹ | Thaila Fernanda dos Reis¹ | Mario Henrique Paziani¹ | Márcia Regina Von Zeska Kress¹ | Diego M. Riaño-Pachón³ | Daisuke Hagiwara⁴ | Laure N. A. Ries¹ | Neil Andrew Brown² | Gustavo H. Goldman¹¹ Faculdade de Ciências Farmacêuticas de Ribeirão Preto, Universidade de São Paulo, Ribeirão Preto, Brazil² Plant Biology and Crop Science, Rothamsted Research, Harpenden, Hertfordshire, UK³ Laboratório Nacional de Ciência e Tecnologia do Bioetanol (CTBE), Centro Nacional de Pesquisa em Energia e Materiais (CNPEM), São Paulo, Brazil⁴ Medical Mycology Research Center, Chiba University, Chiba, Japan

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Abstract

Invasive aspergillosis is predominantly caused by *Aspergillus fumigatus*, and adaptations to stresses experienced within the human host are a prerequisite for the survival and virulence strategies of the pathogen. The central signal transduction pathway operating during hyperosmotic stress is the high osmolarity glycerol mitogen-activated protein kinase cascade. A *fumigatus* MpkC and SakA, orthologues of the *Saccharomyces cerevisiae* Hog1p, constitute the primary regulator of the hyperosmotic stress response. We compared A. *fumigatus* wild-type transcriptional response to osmotic stress with the Δ mpkC, Δ sakA, and Δ mpkC Δ sakA strains. Our results strongly indicate that MpkC and SakA have independent and collaborative functions during the transcriptional response to transient osmotic stress. We have identified and characterized null mutants for four A. *fumigatus* basic leucine zipper proteins transcription factors. The *atfA* and *atfB* have comparable expression levels with the wild-type in Δ mpkC but are repressed in Δ sakA and Δ mpkC Δ sakA post-osmotic stress. The *atfC* and *atfD* have reduced expression levels in all mutants post-osmotic stress. The *atfA-D* null mutants displayed several phenotypes related to osmotic, oxidative, and cell wall stresses. The Δ atfA and Δ atfB were shown to be avirulent and to have attenuated virulence, respectively, in both *Galleria mellonella* and a neutropenic murine model of invasive pulmonary aspergillosis.

1 | INTRODUCTION

Invasive aspergillosis has emerged as one of the most common life-threatening fungal diseases of immunocompromised humans and has been reported to have mortality rates as high as 90% (Brakhage, 2005; Brown, Denning & Levitz, 2012a; Brown, Denning, Gow, Levitz, & Netea et al., 2012b; Lackner and Lass-Flörl, 2013). Invasive aspergillosis is predominantly caused by *Aspergillus fumigatus*, a saprophytic, soil-dwelling filamentous fungus that produces conidia as means of dispersal that can be inhaled by humans. A. *fumigatus* is a major opportunistic pathogen and allergen of mammals (Greenberger, 2002; Dagenais and Keller, 2009), causing approximately 65% of all invasive fungal infections in humans while being the most frequently encountered species accounting for fungal pulmonary infections

(Brown et al., 2012a; Brown et al., 2012b; Lackner & Lass-Flörl, 2013). The ability to sense and adapt to the surroundings is essential for the survival of A. *fumigatus* within a mammalian host. A. *fumigatus* adaptations to stresses experienced within the human host are a prerequisite for the survival and virulence strategies of the pathogen (Brown & Goldman, 2016). There is a general consensus that aspergillosis is a multifactorial disease with several phenotypes influencing the final outcome of the disease establishment (Tekaiia & Latgé, 2005; Hartmann et al., 2011; Sugui, Kwon-Chung, Juvvadi, Latgé, & Steinbach, 2014).

The highly conserved mitogen-activated protein kinase (MAPK) signaling pathways are essential for adaptations to environmental change (Pearson et al., 2001; Rispaill et al., 2009). The MAPK cascades are important for relaying, integrating, and amplifying

intracellular signals and are crucial signaling components involved in many cellular processes (Pearson et al., 2001; Rispail et al., 2009). The central signal transduction pathway operating during hyperosmotic stress is the high osmolarity glycerol (HOG) MAPK cascade (Martínez-Montañés, Pascual-Ahuir, & Proft 2010; de Nadal & Posas, 2015). In *Saccharomyces cerevisiae*, the Hog1p MAPK is involved in many aspects of the osmotic stress adaptation, modulating ion transport across cell membranes, regulating cell cycle progression during stress, and affecting translation efficiency, and it is responsible for promoting a complex transcriptional program in the nucleus (Martínez-Montañés et al., 2010; de Nadal & Posas, 2015). In *A. fumigatus*, MpkC and SakA are orthologues of the *S. cerevisiae* Hog1p and constitute the main regulators of the osmotic stress response (OSR; Rispail et al., 2009). The MpkC protein sequence is very similar to that of SakA (68.4% identity; 82.9% similarity; BLAST e-value: 2e-147). SakA and MpkC have also been shown to play a role in caspofungin adaptation and carbon source utilization, respectively (Reyes, Romans, Nguyen, & May, 2006; Altwasser et al., 2015; Valiente, Macheleidt, Föge, & Brakhage, 2015a).

We have recently shown the multifunctional roles of the two *A. fumigatus* Hog1p orthologues, MpkC and SakA (Bruder Nascimento et al., 2016). Both MAPKs were shown to be important for adaptations to osmotic and oxidative stresses, cell wall damage, and heat shock. The simultaneous disruption of both MAPKs had an additive effect, as the $\Delta mpkC \Delta sakA$ double mutant showed increased sensitivity to the aforementioned stresses in comparison with the single $\Delta mpkC$ and $\Delta sakA$ mutants. Furthermore, this interaction was essential for full virulence and macrophage recognition. In the majority of cases, the stress phenotypes of $\Delta mpkC$ were moderate, while the phenotypes of $\Delta sakA$ were more dramatic (Bruder Nascimento et al., 2016). Because most of the phenotypes observed for $\Delta mpkC$ were milder than the $\Delta sakA$ mutant, we proposed that both the cell wall integrity (CWI) and HOG pathways collaborate to cope with several different types of stress and cell wall biosynthesis and that MpkC could act by modulating SakA activity.

The basic leucine zipper proteins (b-ZIP) transcription factors are characterized by the presence of a coiled-coil motif of seven amino acids that mediates dimerization (Vinson, Acharya, & Taparowsky, 2006). Actually, this dimerization domain was named leucine zipper because there is a leucine residue for every seven amino acids (Vinson et al., 2006). In b-ZIP, the DNA binding motif is positioned immediately N-terminal to the dimerization domain (Landschultz et al., 1988; Vinson et al., 2006). b-ZIP proteins can potentially form homodimers and heterodimers that can have different biological functions. The *Schizosaccharomyces pombe* Atf1p was first identified as a transcription factor containing a b-ZIP domain at the C-terminus with high identity to the mammalian Activating transcription factor/cAMP response element binding protein (ATF/CREB) transcription factors binding specifically to Activating transcription Factor/cyclic AMP Response Element (ATF/CRE) recognition sites (Takeda et al., 1995). Atf1 has been shown to be involved in sexual development, entry into stationary phase, and G1 arrest of the cell cycle, plus osmotic and ultraviolet stress responses (Takeda et al., 1995; Shiozaki & Russell, 1996; Degols & Russell, 1997). In *S. pombe*, mutants defective in the HOG MAPK Sty1/Spc1-Atf1 regulatory pathway are hypersensitive to different types of environmental

stresses (Nguyen, Lee, Place, & Shiozaki, 2000; Quinn et al., 2002; Chen et al., 2008). Takada et al. (2007) demonstrated that $\Delta atf1$ is hypersensitive to cell wall-damaging agents and that Atf1p is a key component of the *S. pombe* CWI Pmk1 pathway.

Hagiwara, Suzuki, Kamei, Gonoi, and Kawamoto (2014) identified four *A. fumigatus* Atf1p orthologues, named *atfA-D*, and showed that conidia from $\Delta atfA$ mutants were more sensitive to high temperature and oxidative stress, having a reduced trehalose content. Interestingly, these authors also showed that the $\Delta sakA$ and $\Delta mpkC$ mutants both produced normal conidia, while $\Delta sakA \Delta mpkC$ double mutant was defective in conidia stress tolerance, strongly indicating that two MAPKs redundantly regulate the conidia-related function of AtfA. In *Aspergillus nidulans*, the AtfA orthologue is involved in the response to oxidative, osmotic, and heat stresses and regulates development (Hagiwara, Asano, Yamashino, & Mizuno, 2008; Balázs et al., 2010; Lara-Rojas, Sánchez, Kawasaki, & Aguirre, 2011). In *Aspergillus oryzae*, AtfA and AtfB have been reported to be involved in the control of conidial germination and stress tolerance (Sakamoto et al., 2008; Sakamoto et al., 2009).

The present study investigated the early transcriptional response of *A. fumigatus* to osmotic stress. More significantly, we also compared the *A. fumigatus* wild-type transcriptional response to osmotic stress with that of the $\Delta mpkC$, $\Delta sakA$, and $\Delta mpkC \Delta sakA$ mutant strains. This approach revealed how *A. fumigatus* adapted to osmotic stress, highlighting several putative signal transduction pathways and transcription factors important for this adaptation that appear transcriptionally dependent on MpkC and SakA. Subsequent genetic analyses showed the importance of AtfA-D to the adaptation to osmotic, oxidative, and cell wall stresses, in addition to fungal virulence.

2 | RESULTS

2.1 | Transcriptome analysis of the wild-type, $\Delta mpkC$, $\Delta sakA$, and $\Delta mpkC \Delta sakA$ strains exposed to osmotic stress

RNA sequencing was used to interrogate how *A. fumigatus* adapts to hyperosmotic conditions. In order to identify which genes were dependent on MpkC and SakA, we evaluated the genome-wide transcriptional profile of the wild-type, $\Delta mpkC$, $\Delta sakA$, and $\Delta mpkC \Delta sakA$ strains before and after exposure to 1 M sorbitol for 10 min. We defined the differentially expressed genes as those with a minimum of twofold change in gene expression (controlling at a false discovery rate [FDR] of 0.05) when compared with the unstressed equivalent of the same strain (Figure 1a, control, 0 min; Figure S1; Tables S1–S10). In the wild-type strain, 962 and 731 genes were upregulated and downregulated, respectively, post-osmotic stress (17.2% of a total of 9840 genes). Gene ontology (GO) enrichment analyses for the wild-type strain demonstrated a transcriptional upregulation of genes encoding proteins involved in carbohydrate, polyol, lipid, and trehalose metabolic processes. Conversely, there was a downregulation of genes encoding for proteins involved in primary, nucleic acid, and nitrogen compound metabolic processes, accompanied by a repression of ribosome biogenesis, RNA processing,

FIGURE 1 Transcriptome analysis shows that *Aspergillus fumigatus* MpkC and SakA have different and complementary functions. (a) Global distribution of the RNA-seq data for the wild-type exposed to 10 min sorbitol 1.0 M. (b) Venn diagram comparing the genes specifically expressed in each mutant strain relative to the wild-type strain

and DNA-dependent DNA replication initiation (Table 1, first column; Table S11). This initial analysis of the wild-type transcriptome implies that *A. fumigatus* downregulates growth and protein translation while increasing the degradation of lipids and shifting primary metabolism to the production of osmolytes and trehalose.

The genes that were differentially expressed in the three MAPK mutants post-exposure to 1 M sorbitol for 10 min were identified ($\log_2FC \geq 1.0$ and ≤ -1.0 , FDR of 0.05). Among the three MAPK mutants, $\Delta mpkC$ showed a lower number of transcriptional modulations than the $\Delta sakA$ and $\Delta mpkC \Delta sakA$ mutants. Venn analysis revealed a complex pattern of shared and mutant-specific transcriptional responses to osmotic stress (Figure 1b and Table S13). A small proportion of the transcriptional response was shared by all three MAPK mutants, when compared with the wild-type strain, including 240 and 167 upregulated or downregulated genes. The conservation of modulated genes in the MAPK mutants was low between the individual $\Delta sakA$ and $\Delta mpkC$ mutants, including 258 and 199 upregulated or downregulated genes, while each mutant demonstrated a high number of strain-specific transcriptional responses. However, the conservation in gene modulation was high between $\Delta sakA$ and $\Delta mpkC$ with $\Delta mpkC \Delta sakA$, including 796/412 and 1042/329 upregulated or downregulated genes, while the $\Delta mpkC \Delta sakA$ mutant

showed the highest number of strain-specific transcriptional modulations, including 518 upregulated and 402 downregulated genes. This suggests that SakA and MpkC have different and overlapping roles in the osmotic stress response, transcriptionally activating and repressing osmo-dependent genes, while the double MAPK mutation has the greatest impact.

The transcriptomes of the three mutants post-osmotic stress were individually compared to the wild-type strain ($\log_2FC \geq 1.0$ and ≤ -1.0) revealing (a) from the 962 genes upregulated in the wild-type, there were 132, 655, and 710 genes that were not induced to wild-type levels in the $\Delta mpkC$, $\Delta sakA$ and $\Delta mpkC \Delta sakA$ mutants, respectively, and (b) from the 731 genes downregulated in the wild-type, there were 158, 218, and 468 genes that were not repressed to wild-type levels in the $\Delta mpkC$, $\Delta sakA$ and $\Delta mpkC \Delta sakA$ mutants, respectively (Table S12).

Gene ontology enrichment analyses revealed the distinct biological functions of the transcriptional differences between the strains. The 158 genes that were not repressed to wild-type levels in the $\Delta mpkC$ strain post-osmotic stress showed the induction of genes involved in mitotic cell cycle, nucleic acid metabolism, primary metabolic process, and ribosome biogenesis (Tables 2 and S11). There was no enrichment for the 132 genes that were not repressed to wild-type levels in $\Delta sakA$ were involved in cellular response to oxidative and osmotic stresses, trehalose catabolic process, and two-component signal transduction (Tables 3 and S11). The 218 genes that were not repressed to wild-type levels in $\Delta sakA$ were involved in mitotic cell cycle, nucleic acid metabolic process, primary metabolic process, and ribosome biogenesis (Tables 3 and S11).

Finally, GO enrichment analyses for the 710 genes that were not induced to wild-type levels in $\Delta mpkC \Delta sakA$ revealed the repression of genes involved in cellular carbohydrate catabolic process, osmosensory signaling pathway, response to heat, and trehalose metabolic process (Tables 4 and S12). In contrast, the 468 genes that were not repressed to wild-type levels in $\Delta mpkC \Delta sakA$ were involved in DNA-dependent DNA replication initiation, metabolic process, nucleic acid metabolic process, ribosome biogenesis, cellular carbohydrate catabolic process, osmosensory signaling pathway, response to heat, and trehalose metabolic process (Tables 4 and S12).

The mutant transcriptomes were also compared with the wild-type strain in the absence of osmotic stress (time zero, control; $\log_2FC \geq 1.0$ and ≤ -1.0 , FDR of 0.05; Tables 5 and S14). A comparison of the genes specifically induced or repressed at time zero with those modulated post-10 min osmotic stress, in each mutant strain relative to the wild-type, revealed a substantial overlap between the two timepoints (Table S14). GO enrichment analyses for the genes downregulated in the mutants at both time zero and post-10 min osmotic stress showed the repression of genes involved in the response to oxidative stress and cellular polysaccharide catabolic process (in $\Delta mpkC$, $\Delta sakA$, and $\Delta mpkC \Delta sakA$) and hyperosmotic response (in $\Delta mpkC \Delta sakA$; Table S15). In contrast, the genes upregulated in the mutants were involved in ribosome biogenesis, nuclear division, and carboxylic organic acids (in $\Delta mpkC$, $\Delta sakA$, and $\Delta mpkC \Delta sakA$) and DNA and RNA metabolic processes (in $\Delta mpkC \Delta sakA$; Table S15).

TABLE 1 Comparison of GO process categories that are significantly enriched upon osmotic stress in *Aspergillus fumigatus* (Af), *Saccharomyces cerevisiae* (Sc), *Schizosaccharomyces pombe* (Sp), and *Candida albicans* (Ca)

GO process Down	Description	p-value (observed genes/total number of genes)			
		Af	Sc	Sp	Ca
51276	Chromosome organization		1.74e-07 (209/1875)		
6325	Chromatin organization		2.94e-07 (128/1875)		
51169	Nuclear transport	0.0015 (18/731)	5.02e-07 (89/1875)		1.07e-20 (73/935)
90304	Nucleic acid metabolic process	1.39E-19 (139/731)	2.08e-49 (607/1875)		7.11e-91 (384/935)
16070	RNA metabolic process	3.62E-27 (132/731)	1.96e-49 (521/1875)	1.23E-05 (185/891)	1.09e-96 (327/935)
6259	DNA metabolic process		8.06e-11 (184/1875)	1.63E-03 (84/891)	1.15e-06 (97/935)
6260	DNA replication			5.95E-04 (43/891)	
6267	Pre-replicative complex assembly	3.54E-07 (9/731)			
1903047	Mitotic cell cycle process			1.38E-03 (89/891)	
51320	S phase	0.0007 (6/731)			
84	S phase of mitotic cell cycle	0.0002 (6/731)			
7049	Cell cycle				3.46e-06 (97/935)
278	Mitotic cell cycle			1.13E-03 (90/891)	9.70e-06 (88/935)
42254	Ribosome biogenesis	1.11E-51 (101/731)	3.47e-26 (175/1875)		4.11e-107 (201/935)
6520	Cellular amino acid metabolic process		1.05e-14 (77/1875)		
6629	Lipid metabolic process			3.60E-03 (66/891)	
6793	Phosphorus metabolic process		2.57e-08 (221/1875)		
6807	Nitrogen compound metabolic process	4.21E-14 (175/731)	1.47e-63 (942/1875)	1.37E-02 (352/891)	1.52e-120 (582/935)
16192	Vesicle-mediated transport		6.21e-07 (155/1875)	1.52E-02 (85/891)	
UP		Af	Sc	Sp	Ca
44238	Primary metabolic process		3.40e-05 (323/549)	5.92E-10 (761/1352)	1.38e-55 (977/1707)
51186	Cofactor metabolic process		0.00013 (36/549)	1.82E-04 (83/1352)	2.92e-10 (90/1707)
5991	Trehalose metabolic process	0.00008 (7/962)			
5993	Trehalose catabolic process	0.00009 (5/962)			
6066	Alcohol metabolic process	0.0003 (33/962)			0.01745 (40/1707)
9311	Oligosaccharide metabolic process	0.001 (33/962)			
46352	Disaccharide catabolic process	0.00009 (5/962)			
44262	Cellular carbohydrate metabolic process	0.00004 (43/962)			
16139	Glycoside catabolic process	0.00009 (5/962)			
19751	Polyol metabolic process	0.0006 (10/962)			
15980	Energy derivation by oxidation of organic compounds			8.61E-03 (32/1352)	
1901135	carbohydrate derivative metabolic process			6.74E-03 (107/1352)	4.41e-05 (125/1707)
6091	generation of precursor metabolites and energy			1.05E-03 (45/1352)	
16053	organic acid biosynthetic process			1.13E-03 (72/1352)	
19752	carboxylic acid metabolic process (GO:0019752)			1.15E-07 (129/1352)	
71704	organic substance metabolic process				2.66e-66 (1063/1707)
6082	organic acid metabolic process			1.25E-09 (146/1352)	0.00044 (147/1707)
44281	small molecule metabolic process		3.30e-10 (105/549)	2.30E-19 (267/1352)	
6508	Proteolysis		1.51e-15 (70/549)		

(Continues)

TABLE 1 (Continued)

GO process Down	Description	p-value (observed genes/total number of genes)			
		Af	Sc	Sp	Ca
6511	ubiquitin-dependent protein catabolic process		1.15e-15 (56/549)		7.57e-28 (121/1707)
6950	response to stress		0.00906 (82/549)		7.77e-15 (324/1707)

GO, gene ontology; OSR, osmotic stress response.

Functional categories for these species were identified using GO resources at Aspgd (www.aspgd.org), SGD (www.yeastgenome.org/GOContents.shtml), PomBase (<http://www.pombase.org/>), and CGD (<http://www.candidagenome.org/>). We have removed redundant categories that have groups with weak probability and apparently are not participating in the response. *Saccharomyces cerevisiae* OSR genes were from O'Rourke and Herskowitz (2004; 0.5 M KCl for 10 min), *Schizosaccharomyces pombe* OSR genes were from Chen et al. (2003; 1 M sorbitol for 15 min), and *Candida albicans* OSR genes were from Enjalbert et al. (2006; 0.3 M NaCl for 10 min).

For the full list, refer to Tables S11 and S17.

TABLE 2 A summary of the overrepresented GO terms upregulated in $\Delta mpkC$ versus wild-type post-transfer to 1 M sorbitol for 10 min

GO term	Description	p-value	Class	Regulation	Genes
GO:0000278	Mitotic cell cycle	0.001219971	BP	Up	11
GO:0090304	Nucleic acid metabolic process	3.42349E-15	BP	Up	56
GO:0006267	Pre-replicative complex assembly	1.9047E-05	BP	Up	5
GO:0044238	Primary metabolic process	0.001357829	BP	Up	68
GO:0042254	Ribosome biogenesis	6.32972E-38	BP	Up	51

For the full list, refer to Table S11.

TABLE 3 - A summary of the overrepresented gene ontology terms among the genes that were upregulated or downregulated in $\Delta sakA$ versus wild-type post-transfer to 1 M sorbitol for 10 min

GO term	Description	p-value	Class	Regulation	Genes
GO:0071470	Cellular response to osmotic stress	0.000535	BP	Down	10
GO:0006979	Response to oxidative stress	0.000178	BP	Down	17
GO:0005993	Trehalose catabolic process	0.000392	BP	Down	4
GO:0000160	Two-component signal transduction system	0.000428	BP	Down	5
GO:0000278	Mitotic cell cycle	0.00122	BP	Up	11
GO:0090304	Nucleic acid metabolic process	3.42E-15	BP	Up	56
GO:0006267	Pre-replicative complex assembly	1.9E-05	BP	Up	5
GO:0044238	Primary metabolic process	0.001358	BP	Up	68
GO:0042254	Ribosome biogenesis	6.33E-38	BP	Up	51

GO, gene ontology.

For the full list, refer to Table S11.

TABLE 4 A summary of the GO terms overrepresented upregulated or downregulated in $\log_2FC \Delta mpkC \Delta sakA$ versus wild-type post-transfer to 1 M sorbitol for 10 min

GO term	Description	p-value	Class	Regulation	Genes
GO:0006270	DNA-dependent DNA replication initiation	0.001211	BP	Up	7
GO:0008152	Metabolic process	0.001273	BP	Up	185
GO:0051168	Nuclear export	0.000352	BP	Up	14
GO:0090304	Nucleic acid metabolic process	9.67E-27	BP	Up	130
GO:0006267	Pre-replicative complex assembly	1.66E-05	BP	Up	7
GO:0042254	Ribosome biogenesis	3.95E-62	BP	Up	100
GO:0044275	Cellular carbohydrate catabolic process	0.000451	BP	Down	16
GO:0007231	Osmosensory signaling pathway	0.0007	BP	Down	7
GO:0009408	Response to heat	0.00061	BP	Down	12
GO:0005991	Trehalose metabolic process	0.000206	BP	Down	6

GO, gene ontology.

For the full list, refer to Table S14.

TABLE 5 Number of genes specifically expressed in the time zero (control) and time 10 min (sorbitol 1 M) in the $\Delta sakA$, $\Delta mpkC$, and $\Delta mpkC \Delta sakA$ mutant strains

Treatment	Overlap time zero $\times$ time 10 min	Time zero uniques $\Delta sakA$	Time 10 min uniques $\Delta sakA$	Time zero uniques $\Delta mpkC$	Time 10 min uniques $\Delta mpkC$	Time zero $\Delta mpkC \Delta sakA$	Time 10 min $\Delta mpkC \Delta sakA$
induced (640) $\Delta sakA$ time zero $\times$ induced (1245) $\Delta sakA$ 10 min	479	161	766				
repressed (1065) $\Delta sakA$ time zero $\times$ repressed (1383) $\Delta sakA$ 10 min	696	369	685				
induced (317) $\Delta mpkC$ control $\times$ induced (664) $\Delta mpkC$ 10 min	203			114	461		
repressed (543) $\Delta mpkC$ control $\times$ repressed (668) $\Delta mpkC$ 10 min	317			226	351		
induced (778) $\Delta mpkC \Delta sakA$ control $\times$ induced (1486) $\Delta mpkC \Delta sakA$ 10 min	628					150	858
repressed (1243) $\Delta mpkC \Delta sakA$ $\times$ repressed (1606) $\Delta mpkC \Delta sakA$ 10 min	823					420	782

For the full list, refer to Table S15.

Finally, genes that were differentially expressed in the mutants compared with the wild-type strain only at time zero were identified (Tables S14 and S15). There were 161, 114, and 150 genes upregulated in $\Delta mpkC$, $\Delta sakA$, and $\Delta mpkC \Delta sakA$ mutants, respectively, at time zero (Tables 5 and S14). We observed 369, 226, and 420 genes that were downregulated in $\Delta mpkC$, $\Delta sakA$, and $\Delta mpkC \Delta sakA$ mutants, respectively, at time zero (Tables 5 and S14). Because these genes were not modulated in the wild-type strain upon osmotic stress, their differential expression suggests that MpkC and SakA perform biological roles that are not exclusively related to osmotic stress. We were not able to see any enrichment for GO terms within the genes upregulated and downregulated in the $\Delta sakA$ and $\Delta mpkC \Delta sakA$ mutants and upregulated in the $\Delta mpkC$ mutant at time zero (Table S15). Interestingly, most of these genes encoded for unknown, but fungal conserved, proteins (Tables S1–S10, S14, and S15). However, there was an enrichment for genes downregulated in the $\Delta mpkC$ mutant at time zero encoding for proteins involved in cellular carbohydrate, carboxylic, organic acid, and alcohol metabolic processes (Table S15).

Therefore, this functional analysis of the strains transcriptomes highlighted the distinct and complementary functions of MpkC and SakA in the OSR. Interestingly, our results indicate that SakA and MpkC perform distinct and complementary biological roles during normal growth and not only upon osmotic stress.

2.2 | Comparison of *A. fumigatus* transient osmotic stress responses with other fungi

The present *A. fumigatus* osmotic stress response transcriptomic dataset was then compared with comparative transcriptomic datasets from other model fungi, such as *S. cerevisiae*, *S. pombe*, and *Candida albicans* (Sc-Sp-Ca dataset obtained from Enjalbert et al., 2006, and summarized in Table S16), exposed to osmotic transient stress with 0.5 M KCl for 10 min, 1 M sorbitol for 15 min, and 0.3 M NaCl for 10 min, respectively (O'Rourke & Herskowitz, 2004; Chen et al., 2003; Enjalbert et al., 2006). First, we compared the functional categories that were significantly enriched for all four datasets (Tables 1 and S17). In all datasets, DNA and RNA metabolism, cell cycle progression,

and nitrogen compound metabolic processes were decreased (Tables 1 and S17). However, genes involved in ribosome biogenesis were only downregulated in *A. fumigatus* and *S. cerevisiae*, while vesicle-mediated transport was only downregulated in *S. cerevisiae* and *S. pombe* (Tables 1 and S17). Interestingly, cellular amino acid and phosphorus and lipid metabolic processes were only downregulated in *S. cerevisiae* and *S. pombe*, respectively (Tables 1 and S17). In all four fungal species, genes that encode proteins involved in primary metabolism, carbohydrate metabolism, trehalose, polyol, and organic substances were upregulated (Tables 1 and S17). The upregulation of proteolysis and ubiquitin-dependent protein catabolic process was unique to *S. cerevisiae* and *C. albicans* (Tables 1 and S17). Surprisingly, response to stress was only upregulated in *S. cerevisiae* and *C. albicans* (Tables 1 and S17).

Subsequently, we compared *A. fumigatus* genes modulated during osmotic stress (AfOS) with the Sc-Sp-Ca dataset containing 753 genes that show dependence on HOG1 (as defined by Enjalbert et al., 2006; see Experimental procedures and Table S18). Twenty-nine *A. fumigatus* genes have no orthologues in this enriched dataset (Table S18). Table S18 shows that 77.8% (44.8% and 33.0% upregulated and downregulated, respectively) of the *A. fumigatus* SakA-dependent AfOS response was also HOG1-dependent in Sc-Sp-Ca. About 16.5% of AfOS orthologues present in the enriched Sc-Sp-Ca dataset have non-modulation neither in $\Delta mpkC$, $\Delta sakA$, $\Delta mpkC \Delta sakA$ mutants (Table S18).

Taken together, these results strongly suggest that about 80% of the responses to osmotic stress were common among the four different fungi and dependent on Hog1 or SakA, but there were also species-specific differences.

2.3 | Modulation of genes encoding for proteins involved in signal transduction and transcription factors

We concentrated our attention on network-related signaling proteins important to the OSR. However, because the two-component system (TCS) working upstream the HOG pathway in *Aspergilli* has never been elucidated, the putative signaling pathway was based on bioinformatics predictions. The *A. fumigatus* osmotic stress, HOG pathway,

is composed of two signaling modules: (a) the TCS-like phosphorelay module composed of a hybrid sensor kinase (TcsC/NikA), a histidine-containing phosphotransfer (HPT) protein (YpdA), and a response regulator (RR, SskA); and (b) the MAPK module comprising an MAPK kinase kinase (MAPKKK, SskB), an MAPK kinase (MAPKK, PbsB), and two MAPKs (SakA and MpkC). We evaluated the expression of all members of the two modules (Figure 2a). The genes encoding PhkA (Afu3g12550), PhkB (Afu3g12530), Fhk5 (Afu4g00660), Fhk6 (Afu4g00320), FphB (Afu6g09260), and YpdA (Afu4g10280) were upregulated in the wild-type strain but repressed in all mutants post-exposure to osmotic stress (Figure 2a). Conversely, *tcsB* (Afu2g00660), *fkh4* (Afu4g07400), and *nikA/tcsC* (Afu2g03560) were downregulated in the wild-type strain but upregulated in all three mutants post-exposure to osmotic stress (Figure 2a). In the MAPK module, the genes encoding for Pbs2

(Afu1g15950) and SakA (Afu1g12940) were upregulated in the wild-type strain but downregulated in $\Delta mpkC$ and absent in $\Delta sakA$ and $\Delta mpkC \Delta sakA$ (Figure 2a). Interestingly, SskB (Afu1g10940) and SskA (Afu5g08390) were not transcriptionally modulated by osmotic stress. These results suggest that MpkC and SakA influence the expression of genes in both signaling modules important for their own activation.

Next, we evaluated genes that encode other signal transduction proteins beyond the HOG pathway and transcription factors (Figure 2b and c). In the wild-type strain, there was an upregulation of several protein kinase encoding genes, such as CmkD (Afu2g03490) calcium calmodulin, Ack (Afu3g10750) acetate kinase, HalA (Afu5g08480) a halotolerance kinase (De Souza et al., 2013), SchA (Afu1g06400) phosphorylated by Tor1p and required for target of rapamycin complex 1 (TORC1)-mediated regulation of ribosome

FIGURE 2 The expression of genes encoding proteins putatively involved in the two-component system, the high osmolarity glycerol mitogen-activated protein kinase module, other signal transduction pathways and downstream transcription factors upon osmotic stress. The RNA-seq data for annotated genes corresponding to (a) the two-component system and high osmolarity glycerol mitogen-activate protein kinase module, (b) signal transduction proteins, and (c) transcription factors. The wild-type is shown as 10 min osmotic stress 1 M sorbitol versus time zero (16 hr growth), and gene deletion strains are shown as the deletion strain versus the equivalent wild-type 10 min time point (the mutant values have been normalized to the basal level of expression of each gene before stress, i.e. expression ratios are being compared: wild-type 10 min versus time zero divided by a specific mutant 10 min versus time zero). MeV analysis was performed using hierarchical clustering, using Pearson correlation with complete linkage clustering

biogenesis (Swinnen, Ghillebert, Wilms, & Winderickx, 2014), the regulatory (PkaR) and catalytic (PkaC) subunits of protein kinase A (Afu3g10000 and Afu2g12200), and the putative Ksp1 kinase that associates with TOR1 (Afu5g06470; De Souza et al., 2013). We have also observed in the wild-type strain an upregulation of several genes encoding protein phosphatases, such as PpmA (Afu8g04580) and PypA (Afu4g04710), and G-protein coupled receptors, such as GprF (Afu5g04100) and Afu7g01430 encoding a putative bacterial rhodopsin, and the G-protein alpha subunit GpaB (Afu1g12930). All these genes were downregulated in the three mutant strains (Figure 2b). The protein kinase NimA, a positive mitotic activator (Afu6g02670), the CdcA phosphatase (Afu3g12250), and the G-protein coupled receptor GprM (Afu7g05300) are downregulated in the wild-type and upregulated in all three mutant strains (Figure 2a). Taken together, these results suggest that MpkC and SakA can influence the signal transduction pathways related to protein kinase A, calcium calmodulin, and Target Of Rapamycin (TOR).

Subsequently, we identified putative transcription factors, which were differentially expressed in the wild-type and mutant strains post-exposure to osmotic stress, by visual inspection of the current annotation and further BlastP analysis (Figure 2c and Table 6). This revealed *atfA*, B, C, and D, the orthologues of *S. pombe* Atf-CREB family Atf1 (Gao, Davidson, & Wahls, 2008), to be induced in the wild-type strain. The *atfA* (Afu3g11330) and *atfB* (Afu5g12960) have comparable expression levels with the wild-type strain in the $\Delta mpkC$ mutant but were not induced in the $\Delta sakA$ and $\Delta mpkC \Delta sakA$ mutants post-osmotic stress. The *atfC* (Afu1g17360) and *atfD* (Afu6g12150) have reduced expression levels in all mutants post-osmotic stress. Additionally, several uncharacterized transcription factors were shown to be differentially modulated between strains (Table 6). Recently, Hagiwara et al. (2014) have shown that the *atfA* null mutant conidia was more sensitive to high temperature and oxidative stress and that MpkC and SakA were involved in the regulation of the conidia-related function of AtfA in *A. fumigatus*. Considering the genetic interaction between MpkC/SakA and AtfA, we decided to characterize in more detail AtfA, B, C, and D.

2.4 | A small ATF1 transcription factor family is dependent on MpkC and SakA and is involved in responses to osmotic, cell wall, and oxidative stresses

As previously shown by Hagiwara et al. (2014), *A. fumigatus* has four Atf family proteins, AtfA, -B, -C, and -D (amino acid identity ranges from 58.7% to 65.1%; BLAST e-values from $1e-09$ to $2e-06$), and these authors investigated the role of AtfA in modulating stress tolerance in *A. fumigatus* conidia. Here, we expanded this investigation by exploring which stress responses were mediated by AtfA-D during mycelial growth and development. A comparison of protein structure and organization of AtfA was performed using the SMART interface (<http://smart.embl-heidelberg.de/>): (a) Atf1_OSA (PF11785), from amino acid 89 to 142, which is required for a wide range of responses and that has been shown to be involved in the OSR; (b) Atf1_HRA (PF11786), from amino acid 163 to 233, involved in meiotic recombination and that has been shown to be necessary and sufficient to activate recombination; (c) Atf1_HRR (PF11787), from amino acid 239 to 311, also

involved in meiotic recombination and that has been shown to be necessary and sufficient to repress recombination; and (d) BRLZ (SM00338), from amino acid 441 to 505, the b-ZIP that contains a basic region mediating sequence-specific DNA binding followed by a leucine zipper region required for dimerization. The identity among AtfA-D is restricted to the BLZR domain at the C-terminus of each protein.

First, we validated that *atfA-D* were transcriptionally induced in the wild-type strain during osmotic stress (10 min 1 M sorbitol) using quantitative real-time polymerase chain reaction (PCR) and independent RNA preparations (Figure 3a-d). In these experiments, *asp7*/Afu4g06670 was used as a normalizer because this gene was consistently expressed in all four strains throughout our RNA-seq investigations of osmotic stress. The expression of *atfA-D* showed a high level of correlation with the RNA-seq data (Pearson correlation from 0.819 to 0.998). The function of *atfA-D* was assessed further by generating single gene deletion strains in the CEA17 background. To ensure that all the deletion strains used throughout this study did not possess additional mutations, we complemented them with a wild-type copy of the corresponding gene. We also used the $\Delta atfA$ and $\Delta atfA::atfA^+$ strains (in the Afs35 background) that were previously constructed by Hagiwara et al. (2014). All five mutants showed comparable vegetative growth and asexual sporulation with the wild-type and complemented strains (data not shown).

Both $\Delta atfA^{Afs35}$ and $\Delta atfA^{CEA17}$ had decreased growth in 1.2 M sorbitol (Figure 4a), while $\Delta atfB-D^{CEA17}$ showed comparable growth with the wild-type strain (data not shown). Alterations in osmolarity can have an impact on the composition and structure of the cell wall (Valiante et al., 2015a; Altwasser et al., 2015; Winkelströter et al., 2015a; Bom et al., 2015; Bruder Nascimento et al., 2016). In accordance, both $\Delta atfA^{Afs35}$ and $\Delta atfA^{CEA17}$ mutants were sensitive to the cell wall-damaging agents calcofluor white and Congo red. In contrast, $\Delta atfB$ was only sensitive to Congo red (Figure 4b and c), while $\Delta atfC-D$ showed comparable growth with the wild-type strain (Figure S2). In *A. fumigatus* the TcsC/NikA histidine kinase, which is upstream of the HOG pathway, is involved in fungicide resistance and osmotic stress tolerance (McCormick et al., 2012; Hagiwara et al., 2013). The impact of phenylpyrrole and dicarboximide fungicides, fludioxonil and iprodione, on the growth of *A. fumigatus* $\Delta atfA-D$ strains was evaluated (Figure 4d and e; data not shown). Both the $\Delta atfA^{Afs35}$ and $\Delta atfA^{CEA17}$ strains were more sensitive to iprodione and fludioxonil, while $\Delta atfB$ and $\Delta atfC$ were only more sensitive to iprodione, when compared with the respective wild-type strain (Figure 4d and e; data not shown).

We also evaluated the sensitivity of these strains to β -1,3-glucan synthase inhibitor caspofungin (Figures 5 and 6). Both $\Delta atfA^{Afs35}$ and $\Delta atfA^{CEA17}$ were more sensitive to caspofungin at low and high concentrations (Figure 5a and b). Interestingly, $\Delta atfB$, $\Delta atfC$, and $\Delta atfD$ showed increased resistance to low caspofungin concentrations (from 0.10 to 0.25 μ g/ml), but they were more sensitive to higher caspofungin concentrations (from 0.50 to 1.0 μ g/ml) (Figure 5c-e), suggesting their participation in the caspofungin paradoxical effect (CPE), where you get fungal growth beyond the minimum inhibitory concentration of caspofungin (Steinbach, Lamoth, & Juwadi, 2015). Therefore, the wild-type CEA17 and $\Delta atfB$ strains showed an expected CPE. However, in the $\Delta atfA$, $\Delta atfC$, and $\Delta atfD$ strains, the CPE appears

TABLE 6 Selection of transcription factors with modulated expression upon osmotic stress that were also dependent on MpkC and/or SakA

	Wild-type 10' × 0	$\Delta mpkC$ logFC×10' Wild-type	$\Delta sakA$ logFC×10' Wild-type	$\Delta mpkC \Delta sakA$ logFC×10 Wild-type	Annotation
Afu1g17360 <i>atfC</i>	5.49	-0.63	-3.65	-3.79	Orthologue of <i>Schizosaccharomyces pombe</i> Atf-CREB family Atf1
Afu6g12160	4.34	NM	-2.26	-3.85	Putative C6 transcription factor
Afu3g11170	4.17	NM	-6.16	-6.18	Orthologues play a role in carotenoid biosynthetic process and circadian rhythm
Afu6g10310	3.92	-1.25	2.02	NM	Has domain(s) with predicted RNA polymerase II transcription factor activity
Afu3g03900	3.54	NM	-4.97	-3.56	Has domain(s) with predicted RNA polymerase II transcription factor activity
Afu5g12960 <i>atfB</i>	3.00	NM	-5.54	-6.66	Orthologue of <i>Schizosaccharomyces pombe</i> Atf-CREB family Atf1
Afu6g12150 <i>atfD</i>	2.58	-1.65	-4.99	-3.93	Orthologue of <i>Schizosaccharomyces pombe</i> Atf-CREB family Atf1
Afu8g04130 <i>farB1</i>	2.43	NM	NM	-1.43	C6 TF with a predicted role in beta-oxidation of fatty acids
Afu2g15680	2.34	1.02	-2.93	-2.21	Orthologue of TOA2. TFI small subunit; involved in the transcriptional activation
Afu3g11330 <i>atfA</i>	2.30	NM	-3.14	-3.78	Orthologue of <i>Schizosaccharomyces pombe</i> Atf-CREB family Atf1
Afu2g13770 <i>flbC</i>	2.26	NM	1.01	NM	C2H2 TF involved in asexual development; calcium induced
Afu7g05080	2.23	-2.02	-2.55	-5.15	Has domain(s) with predicted RNA polymerase II transcription factor activity
Afu1g13750	1.91	-0.53	-2.00	-2.28	C2H2 TF; calcium induced
Afu3g05820	1.82	-0.83	-1.78	-2.82	ZZ type zinc finger domain protein; fungal specific transcription factor
Afu8g05570	1.75	-0.99	-1.31	-2.32	Orthologue of SIN3. component of both the Rpd3S and Rpd3L histone deacetylase
Afu4g03460 <i>srbB</i>	1.73	-0.69	-1.80	-3.12	Putative helix-loop-helix DNA binding domain protein; calcium downregulated
Afu1g03210 <i>flbD</i>	1.73	-1.16	1.07	-0.99	Myb family TF; <i>Aspergillus nidulans</i> orthologue plays a role in conidiophore development
Afu4g00950	1.65	NM	-1.21	-1.54	Orthologue of SRB4. subunit of RNA polymerase II mediator complex
Afu1g04140	1.60	0.46	-2.43	-2.52	Has domain(s) with predicted RNA polymerase II transcription factor activity
Afu6g13680	1.50	-0.49	-2.36	-2.20	Has domain(s) with predicted RNA polymerase II transcription factor activity
Afu5g01900 <i>hsf1</i>	1.45	-0.34	-0.91	-1.70	TF that regulates the response to heat shock
Afu4g10200 <i>rfeF</i>	1.22	-0.84	-1.94	-2.69	Putative TF; calcium-induced
Afu2g05310	1.15	-1.24	1.12	-1.64	Has domain(s) with predicted RNA polymerase II transcription factor activity
Afu3g11380	1.11	-0.69	-1.11	-1.32	Orthologue of the SWI1. subunit of the SWI/SNF chromatin remodeling complex
Afu6g05160 <i>azf1</i>	0.99	-1.57	1.04	0.93	C2H2 TF; transcript repressed by hypoxia
Afu2g17880	-0.99	0.55	1.94	1.34	Has domain(s) with predicted RNA polymerase II transcription factor activity
Afu8g00980	-1.00	-0.99	2.25	1.03	Has domain(s) with predicted RNA polymerase II transcription factor activity
Afu6g01970	-1.11	0.54	1.06	1.13	AreA. Putative GATA-like TF; required for growth on numerous nitrogen sources
Afu3g01640	-1.16	NM	1.69	2.16	Has domain(s) with predicted RNA polymerase II transcription factor activity
Afu5g07530	-1.24	0.87	-1.37	-1.53	Has domain(s) with predicted RNA polymerase II transcription factor activity
Afu2g09890 <i>ndtA</i>	-1.46	-1.18	1.29	1.87	Meiosis-specific TF
Afu8g04050 <i>xprG</i>	-1.47	NM	2.13	1.33	p53-like TF that contains a Ndt80-like DNA-binding domain
Afu4g09080 <i>sebA</i>	-1.48	1.29	0.57	0.51	Putative TF; localizes to the nucleus in response to oxidative stress and heat shock
Afu1g04830 <i>abaA</i>	-1.52	NM	1.45	2.28	TF involved in regulation of conidiation
Afu4g06170	-1.60	1.33	2.26	2.31	Has domain(s) with predicted RNA polymerase II transcription factor activity

(Continues)

TABLE 6 (Continued)

	Wild-type 10' × 0	$\Delta mpkC$ logFC×10' Wild-type	$\Delta sakA$ logFC×10' Wild-type	$\Delta mpkC \Delta sakA$ logFC×10' Wild-type	Annotation
Afu8g06460	-1.72	NM	NM	-0.95	Has domain(s) with predicted RNA polymerase II transcription factor activity
Afu5g03030	-1.73	NM	1.92	1.69	Has domain(s) with predicted RNA polymerase II transcription factor activity
Afu3g02750	-2.00	NM	-1.43	NM	Has domain(s) with predicted RNA polymerase II transcription factor activity
Afu4g04320	-2.06	NM	3.90	1.74	Has domain(s) with predicted RNA polymerase II transcription factor activity
Afu7g02080	-3.14	0.98	2.49	2.72	Has domain(s) with predicted RNA polymerase II transcription factor activity

NM, non-modulated; TF, transcription factor.

to be compromised (Figure 6). Subsequently, we exposed the wild-type strain to low and high caspofungin concentrations simulating the CPE and evaluated the expression of *atfA*, *B*, *C*, and *D* (Figure 6b–e). The *atfA* gene showed a low level of induction at different caspofungin concentrations, while *atfB*, *-C*, and *-D* showed a high level of induction at low and high caspofungin concentrations. These results suggest *atfA-D* could be involved in caspofungin resistance and in the CPE.

The $\Delta atfA^{Afs35}$ conidia previously showed notable sensitivity to oxidative stress (Hagiwara et al., 2014). Here, we evaluated if $\Delta atfA-D$ influenced the sensitivity of mycelia to oxidative stress. Both $\Delta atfA^{Afs35}$ and $\Delta atfA^{CEA17}$ showed increased sensitivity to menadione, paraquat, t-butyl hydroperoxide, and H_2O_2 (Figures S3 and S4). The $\Delta atfB$ strain was only more sensitive to t-butyl hydroperoxide (Figures S3 and S4). Additionally, we also observed that only $\Delta atfA^{Afs35}$ and $\Delta atfA^{CEA17}$ were sensitive to high temperatures (Figure S5).

Collectively, these results strongly indicate that AtfA and AtfB were important for the response to osmotic, oxidative, fungicide, and cell wall stresses, while AtfB and AtfC also influenced the response to iprodione. However, we were unable to assign a functional phenotype to AtfD.

2.5 | AtfA is important for *A. fumigatus* virulence in both *Galleria mellonella* and a low dose murine infection

Two animal models, including (a) *G. mellonella* larvae and (b) a neutropenic murine model of invasive pulmonary aspergillosis, were used to evaluate the importance of AtfA-D to *A. fumigatus* pathogenicity (Figures 7 and 8). In the *G. mellonella* model, infection by either of the two wild-type strains, Afs35 or CEA17, resulted in 100% mortality 6 days post-infection (Figure 7a). However, the $\Delta atfA^{Afs35}$ or $\Delta atfA^{CEA17}$ mutant strains respectively showed only a 10% and 20% mortality 10 days post-infection, which was not statistically different to the phosphate buffer saline (PBS) control according to the Mantel–Cox and Gehan–Brestow–Wilcoxon tests (Figure 7a and b). The $\Delta atfB$ mutant caused 40% mortality 10 days post-infection, which was statistically different to wild-type strain (log-rank Mantel–Cox, p -value = 0.0289, and Gehan–Brestow–Wilcoxon, p -value = 0.0297; Figure 7c). There was no statistical difference between the wild-type strains, $\Delta atfC$ and $\Delta atfD$, and the respective

complemented strains (Mantel–Cox and Gehan–Brestow–Wilcoxon, p -value >0.05; Figure 7d and e).

In the neutropenic murine model of invasive pulmonary aspergillosis, wild-type infection by either Afs35 or CEA17 resulted in 100% and 80% mortality 6 or 5 days post-infection, respectively (Figure 8a and b). The $\Delta atfA^{Afs35}$ mutant caused 30% mortality 15 days post-infection, which was not statistically different to the PBS control according to the Mantel–Cox and Gehan–Brestow–Wilcoxon tests (Figure 8a). In contrast, there are no statistical differences between the wild-type strains, $\Delta atfB$, $\Delta atfC$, and $\Delta atfD$, and the corresponding complemented strains (Mantel–Cox and Gehan–Brestow–Wilcoxon, p -value >0.05; Figure 8b–d).

Fungal burden in the neutropenic murine model of invasive pulmonary aspergillosis was measured by quantitative polymerase chain reaction, showing that the $\Delta atfA^{Afs35}$ and $\Delta atfB$ strains exhibited reduced fungal growth within the lungs compared with the wild-type and the complemented $\Delta atfA::atfA^+$ and $\Delta atfB::atfB^+$ strains (Tukey's multiple comparison test, p -values 0.0052 and 0.0164; Figure 9a and b). There were no statistical differences between the wild-type, $\Delta atfC$, $\Delta atfD$ and the corresponding complemented strains (Figure 9c and d; Tukey's multiple comparison test, p -value >0.05). Taken together, these results clearly demonstrate that AtfA plays an important role in *A. fumigatus* virulence. However, the reduced growth of $\Delta atfB$ in the lungs would suggest that AtfB may also contribute to *A. fumigatus* virulence.

3 | DISCUSSION

Aspergillus fumigatus has four MAPKs: (a) MpkA, the central regulator of the CWI pathway that also plays a role in oxidative stress (Valiante et al., 2015a; Du, Sarfati, Latgé, & Calderone, 2006), (b) MpkB is the putative orthologue of filamentous growth/pheromone response pathway, which remains uncharacterized and (c) MpkC and SakA, orthologues of the *S. cerevisiae* Hog1, which are the main regulators of the HOG response pathway (Rispaill et al., 2009). Previously we identified a genetic interaction between MpkC and SakA to counteract osmotic, oxidative, and high temperature stresses, in addition to the regulation of cell wall biosynthesis (Bruder Nascimento et al., 2016). Furthermore, this interaction was essential for virulence and macrophage recognition. Our results suggested that MpkC could act by

FIGURE 3 In *Aspergillus fumigatus*, *atfA*-D expression is increased during osmotic stress. Quantitative real-time polymerase chain reaction for the *Aspergillus fumigatus atfA*-D genes. The strains were grown for 16 hr at 37°C and transferred to 1 M sorbitol for 10 min. The results are expressed as the abundance of cDNA for a specific gene divided by the abundance of cDNA for the normalizer *asp7* (Afu4g06670). The results are presented as the average ($\pm$ one standard deviation) of three experiments (* $p < 0.001$, as determined by t-tests when compared with the wild-type strain)

modulating SakA activity upon exposure to several different types of stress and during cell wall biosynthesis (Bruder Nascimento et al., 2016). The current study therefore aimed to provide further evidence for this hypothesis. Here, we have investigated how the HOG MAPKs MpkC and SakA regulated the global transcriptional response of *A. fumigatus* to osmotic stress.

In *S. cerevisiae*, environmental change can induce a common transcriptional response, termed the environmental stress response (de Nadal & Posas, 2015). The environmental stress response is

important for redirecting the resources from rapid proliferation to stress protection, and stress-induced genes comprise genes for defense against reactive oxygen species (ROS) and DNA damage, carbohydrate metabolism and energy functions, and stress-repressed genes, mostly translation and ribosome biogenesis (Gasch, Spellman, Kao, Carmel-Harel, et al., 2000; López-Maury, Marguerat, & Bähler, 2008; Martínez-Montañés et al., 2010; de Nadal & Posas, 2015). However, each kind of stress has specific signatures, for instance, the induction of genes encoding ROS detoxifying enzymes during oxidative stress or genes encoding enzymes important for osmolyte biosynthesis.

We have also observed a similar and conserved transcriptional program for the *A. fumigatus* wild-type strain during osmotic stress, with the increased expression of genes encoding for proteins involved in defense against ROS and DNA damage, carbohydrate metabolism and energy functions, and stress-repressed genes, mostly translation and ribosome biogenesis. This program seems to be relatively conserved in fungi because about 80% of the genes that were dependent on SakA in *A. fumigatus* were also dependent on Hog1 in *S. cerevisiae*, *S. pombe*, and *C. albicans*. Transcriptional profiling revealed genes directly and/or indirectly influenced by MpkC and SakA. GO enrichment analyses suggested that MpkC plays a major role in the modulation of the response to DNA metabolism while activating mitochondrial functions and cation transport. In contrast, SakA not only seems to modulate pathways involved in nucleotide, fatty acid, nitrogen and organic acid biosynthesis but was also important for the activation of genes involved in mitochondrial and endoplasmic reticulum functions. Strikingly, the GO analysis revealed complementary and additional functions for the double mutant lacking both MpkC and SakA. For instance, the double mutant specifically demonstrated the increased expression of genes controlling cell cycle progression, by regulating proteins involved in RNA polymerase activities, chromatin binding, and mitotic checkpoints, such as the condensin complex. Conversely, the double mutant showed a decrease in the expression of genes involved in energy reserve metabolic process and osmolytes biosynthesis, which appears complementary to SakA function. These results strongly indicate that MpkC and SakA have independent and overlapping functions in regulating the transcriptional response to osmotic stress. In fact, the expression of several genes in the TCS and MAPK module of the HOG pathway was positively or negatively modulated by MpkC and SakA upon osmotic stress. Further investigations of the regulation of these genes would assist in dissection of osmotic stress signaling in *A. fumigatus*.

However, one aspect that is noticeable is the HOG-dependent modulation of several genes encoding G-protein coupled receptors and the regulatory and catalytic subunits of protein kinase A (PKA). This suggests a possible cross-talk between the cAMP/PKA and HOG MAPK pathways in the activation of the OSR. This cross-talk is also observed in *S. cerevisiae*, but in this organism, the cAMP/PKA pathway represses the two main stress-response transcription factors Msn2 and Msn4 to go to the nucleus (Martínez-Montañés et al., 2010; Gutin, Sadeh, Rahat, Aharoni, & Friedman, 2015). In *S. cerevisiae*, upon osmotic stress, PKA activity is decreased, alleviating Msn2 and Msn4 repression and allowing them to translocate to the nucleus activating stress-responsive genes (Martínez-Montañés et al. 2010). Here, we observed that exposure of *A. fumigatus* to a transient osmotic stress induces a transcriptional accumulation of cAMP/PKA

FIGURE 4 The $\Delta atfA$ and $\Delta atfB$ mutant strains are more sensitive to sorbitol and cell wall-damaging agents. The wild-type, mutant, and complemented strains were grown on MM with (a) sorbitol 1.2 M, (b) calcofluor white 250 $\mu\text{g/ml}$, (c) Congo red 200 $\mu\text{g/ml}$, (d) iprodione 3.5 $\mu\text{g/ml}$, and (e) fludioxonil 0.02 $\mu\text{g/ml}$ for 96 hr at 37°C. The data are expressed as radial growth specific treatment/radial growth control (mm). The results are expressed as average ($\pm$ one standard deviation) of three independent biological repetitions (* $p < 0.001$, determined by t-tests when compared with the wild-type strain)

pathway genes. These results stress a different strategy of gene regulation upon osmotic stress between *A. fumigatus* and *S. cerevisiae*.

Another interesting aspect was the upregulation upon osmotic stress of several genes encoding for protein kinases related to calcium and TOR, such as calcium calmodulin (CmkD), SchA^{SC149} (*S. cerevisiae* functional orthologue of mammalian S6 kinase; phosphorylated by Tor1p and required for TORC1-mediated regulation of ribosome biogenesis, translation initiation, and entry into G0 phase; involved in transactivation of osmotic stress-responsive genes; Swinnen et al., 2014), and Ksp1 (*S. cerevisiae* serine/threonine protein kinase that associates with TORC1 and it is likely involved in TOR signaling cascades; a negative regulator of autophagy; Umekawa & Klionsky, 2012). We have recently characterized the *A. fumigatus* SchA showing that $\Delta schA$ mutant was sensitive to rapamycin, high concentrations of calcium, and hyperosmotic stress (De Castro et al., 2016). The $\Delta schA$ null mutant showed increased phosphorylation of SakA, and as shown here, *schA* was transcriptionally regulated by osmotic stress and this response was dependent on SakA and MpkC. Additionally, the double $\Delta schA \Delta sakA$ and $\Delta schA \Delta mpkC$ mutants were more sensitive to osmotic stress

than the corresponding parental strains (De Castro et al., 2016). Our results strongly suggest there is a complex network of interactions among the *A. fumigatus* TOR, SakA, and SchA pathways.

The expression of the *atfA* and *atfB* transcription factors during osmotic stress in *A. fumigatus* was dependent on SakA, while *atfC* and *atfD* were dependent on both MpkC and SakA. One of these four transcription factors, AtfA, was previously identified by Hagiwara et al. (2014) to influence heat and oxidative stress tolerance. Here, we investigated how these four transcription factors affected mycelial responses to different kinds of stresses including osmotic stress and virulence. The absence of either AtfA or AtfB increases sensitivity not only to osmotic and oxidative stresses but also to cell wall-damaging agents, again suggesting an interconnection between the HOG and CWI pathways. The compromised stress tolerance phenotypes were strongest in the $\Delta atfA$, and moderate in the $\Delta atfB$, strains. Recently, we have shown that $\Delta sakA$ and the double $\Delta mpkC \Delta sakA$ mutants were more sensitive to osmotic and oxidative stresses and to cell wall-damaging agents (Bruder Nascimento et al., 2016). The $\Delta mpkC$, $\Delta sakA$, and $\Delta mpkC \Delta sakA$ mutants were more sensitive to

FIGURE 5 The Δatf mutants are more sensitive to caspofungin. The wild-type and mutant strains (a to e) were grown for 5 days at 37°C. Results are expressed as the average radial diameter of three repetitions ($\pm$ one standard deviation). Statistical analysis was performed by using two-way analysis of variance–Bonferroni. * p -value < 0.001

the β -1,3-glucan synthase and chitin synthase inhibitors, caspofungin and nikkomycin Z, respectively, when compared with the wild-type and complemented strains (Bruder Nascimento et al., 2016). However, the wild-type and the $\Delta mpkC$ strains kept the CPE, while in the $\Delta sakA$ and $\Delta mpkC \Delta sakA$ mutants, the CPE was partially decreased (Bruder Nascimento et al., 2016). Interestingly, $\Delta atfA$ was sensitive to high and low concentrations of caspofungin, while $\Delta atfB$, $-C$, and $-D$ were resistant to low, but sensitive to high, caspofungin concentrations. Additionally, the CPE was only observed in the wild-type and $\Delta atfB$ strains. The expression of $atfB$, $-C$, and $-D$ augmented in response to different caspofungin concentrations, while $atfA$ showed only a discrete increase. It has been proposed that increased concentrations of caspofungin promoted activation and cross-talk between the *A. fumigatus* MpkA and SakA pathways (Altwasser et al., 2015; Bruder Nascimento et al., 2016; Valiante et al., 2015b). Additionally, the existence of a salvage pathway, possibly dependent on MpkC and SakA, which would compensate the caspofungin stress response, has been proposed (Valiante et al., 2015b). This salvage pathway would most likely activate genes (for instance, chitin synthases) that would help to compensate for the reduction of β -1,3-glucan in the cell wall, caused by caspofungin inhibiting β -1,3-glucan synthase activity. We propose that $atfA$ -D are

partially fulfilling this role, either directly or indirectly activating genes that would strengthen the cell wall.

The similar phenotypes observed for the $\Delta sakA$, $\Delta mpkC \Delta sakA$, $\Delta atfA$, and $\Delta atfB$ mutants strongly suggest that AtfA and AtfB could be targets of MpkC/SakA during different kinds of stresses. Hagiwara et al. (2014) have demonstrated that *A. fumigatus* MpkC and SakA were involved in the regulation of the conidia-related function of AtfA. Lara-Rojas et al. (2011) have shown that *A. nidulans* SakA and AtfA were interacting in general stress-signaling pathway important for responding to oxidative or osmotic stress. These authors demonstrated that AtfA was constantly residing in the nucleus, and upon a signaling event, such as oxidative or osmotic stress, SakA is translocated to the nucleus and interacts with AtfA, activating its function as a transcription factor. Preliminary results in our laboratory have shown that *A. fumigatus* AtfA is also constantly located in the nucleus (data not shown), and we are currently investigating if Sak/MpkC could physically interact with AtfA and AtfB. Based on these results, we propose that MpkC and SakA are activating AtfA (and possibly AtfB, $-C$, and $-D$) as a transcription factor upon different kinds of stresses. AtfA is possibly involved in the induction of several genes encoding proteins important for responding and coping to stressing conditions and adapting to the host environment.

FIGURE 6 The $\Delta atfA$, $\Delta atfC$, and $\Delta atfD$ mutants have lost the caspofungin paradoxical effect. (a) The wild-type and mutant strains were grown for 5 days at 37°C. (b to d). In *Aspergillus fumigatus*, *atfA-D* expression is increased upon exposure to caspofungin. Quantitative real-time polymerase chain reaction for the *Aspergillus fumigatus atfA-D* genes. The strains were grown for 24 hr at 37°C in MM and transferred to different concentrations of caspofungin for 30 min. The results are expressed as the abundance of cDNA for a specific gene divided by the abundance of cDNA for the normalizer βtub . The results are presented as the average ($\pm$ one standard deviation) of three experiments (* $p < 0.001$, as determined by t-tests when compared with the wild-type strain)

By using two different animal models of infection, we have shown that *A. fumigatus* $\Delta atfA$ (in two different genetic backgrounds, CEA17 and Afs35) was avirulent, while $\Delta atfB$ had attenuated virulence. Although $\Delta atfC$ showed reduced virulence in the neutropenic murine model of survival, fungal burden did not confirm a reduction in fungal infection. Accordingly, $\Delta atfC$ and $\Delta atfD$ seem to provide a minor contribution to virulence. To our knowledge, ATF1 orthologues have been characterized only in two other human pathogenic fungi, *Cryptococcus neoformans* and *Penicillium marneffei* (Missall & Lodge, 2005; Jung et al., 2015; Kim et al., 2010; Nimmanee, Woo, Vanittanakom, Youngchim, & Vanittanakom, 2014). The *Cryptococcus neoformans* Atf1 orthologue was dependent on carbonic anhydrases and played an important role in several kinds of stress responses, such as thermotolerance, antifungal drug resistance, regulation of thioredoxin expression, repression of melanin and capsule production, and virulence (Missall & Lodge, 2005; Kim et al., 2010; Jung et al., 2015). In the

dimorphic fungus *P. marneffei*, recovery of $\Delta atfA$ mutant conidia after mouse and human macrophage infections was significantly decreased. This strongly suggested that under specific stress conditions, AtfA was required by *P. marneffei*, potentially contributing to the fight against the host immune response during the establishment of infection (Nimmanee et al., 2014).

In summary, we defined the transcriptional landscape of the *A. fumigatus* OSR. Additionally, we identified the specific and synergistic roles of the terminal MAPKs of the HOG pathway, MpkC and Saka, in modulating this response. Once more, we observed a genetic interaction between MpkC and Saka, during exposure to osmotic, oxidative, high temperature, and cell wall-damaging stresses (Bruder Nascimento et al., 2016). Subsequently, we identified four *A. fumigatus* b-ZIP transcription factors, *atfA-D*, as being transcriptionally regulated by MpkC/Saka upon osmotic stress. AtfA was identified as contributing to the *A. fumigatus* virulence, and deletion

FIGURE 7 *Aspergillus fumigatus* AtfA and AtfB contribute to virulence in *Galleria mellonella*. Comparative analysis of wild-type and mutant strains in *Galleria mellonella* animal model. Larvae in groups of 10 per strain were infected with a 5 μ l suspension of conidia at a dose of 1×10^6 /larva. (a) The $\Delta atfA^{CEA17}$ mutant compared with the wild-type and $\Delta atfA^{CEA17}::atfA^+$ strains, (b) the $\Delta atfA^{Afs35}$ mutant compared with the wild-type and $\Delta atfA^{Afs35}::atfA^+$ strains, (c) the $\Delta atfB$ mutant compared with the wild-type and $\Delta atfB::atfB^+$ strains, (d) the $\Delta atfC$ mutant compared with the wild-type and $\Delta atfC::atfC^+$ strains, and (e) the $\Delta atfD$ mutant compared with the wild-type and $\Delta atfD::atfD^+$ strains. PBS = phosphate buffer saline; in (a) and (b) * p -values < 0.001 while in (c) p -values of 0.0289 and 0.0297 for the comparison between the wild-type and $atfB$ null mutant, Log-rank Mantel-Cox and Gehan-Breslow-Wilcoxon tests, respectively

of the corresponding gene resulted in increased sensitivity to osmotic, oxidative, and cell wall-related stresses, suggesting a role for this transcription factor in various cellular processes. This work demonstrates how the analysis of converging signal transduction pathways can be used to identify downstream events fundamental to the regulation of fungal stress tolerance and virulence. Further characterization of the interactions between these transcription factors and the gene targets they regulate will in turn help us to understand how *A. fumigatus* adapts to its environment, within and beyond the host organism.

within individually ventilated cages and were cared for in strict accordance with the principles outlined by the Brazilian College of Animal Experimentation (Princípios Éticos na Experimentação Animal—Colégio Brasileiro de Experimentação Animal, COBEA) and Guiding Principles for Research Involving Animals and Human Beings, American Physiological Society. All efforts were made to minimize suffering. Animals were clinically monitored at least twice daily and humanely sacrificed if moribund (defined by lethargy, dyspnoea, hypothermia, and weight loss). All stressed animals were sacrificed by cervical dislocation.

4 | EXPERIMENTAL PROCEDURES

4.1 | Ethics statement

The principles that guide our studies are based on the Declaration of Animal Rights ratified by the UNESCO in January 27, 1978, in its 8th and 14th articles. All protocols used in this study were approved by the local ethics committee for animal experiments from the Campus of Ribeirão Preto, Universidade de São Paulo (Permit 08.1.1277.53.6; Studies on the interaction of *Aspergillus fumigatus* with animals). All animals were housed in groups of five

4.2 | Strains, media and culture methods

The *A. fumigatus* strains used in this study are described in Table 7. Media were of two basic types. A complete medium (YAG: 2% (w/v) glucose, 0.5% (w/v) yeast extract, 2% (w/v) agar, trace elements) with three variants: YUU (YAG supplemented with 1.2 g/L each of uracil and uridine) and liquid YG or YUU medium of the same composition but without agar. A modified minimal medium (MM: 1% (w/v) glucose, original high nitrate salts, trace elements, 2% (w/v) agar, pH 6.5) was also used. Trace elements, vitamins, and nitrate salts were described by Kafer (1977).

FIGURE 8 *Aspergillus fumigatus* AtfA contributes to virulence in neutropenic mice. Comparative analysis of wild-type and mutant strains in a neutropenic murine model of pulmonary aspergillosis. Mice in groups of 10 per strain were infected intranasally with a 20 μ l suspension of conidia at a dose of 10^5 . (a) The $\Delta atfA^{Afs35}$ mutant compared to the wild-type and $\Delta atfA^{Afs35}::atfA^+$ strains, (b) the $\Delta atfB$ mutant compared with the wild-type and $\Delta atfB::atfB^+$ strains, (c) the $\Delta atfC$ mutant compared with the wild-type and $\Delta atfC::atfC^+$ strains, and (d) the $\Delta atfD$ mutant compared with the wild-type and $\Delta atfD::atfD^+$ strains. PBS = phosphate buffer saline; *p-values 0.1920 and 0.1931 for the comparison between the PBS and *atfA* null mutant, Log-rank Mantel-Cox and Gehan-Breslow-Wilcoxon tests, respectively

FIGURE 9 The $\Delta atfA$ and $\Delta atfB$ mutant strains have reduced fungal burden in murine lungs. Fungal burden was determined 72 hr post-infection by Quantitative real-time polymerase chain reaction based on 18S rRNA gene of *Aspergillus fumigatus* and an intronic region of the mouse GAPDH gene. Fungal and mouse DNA quantities were obtained from the Ct values from an appropriate standard curve. The results are the means ($\pm$ one standard deviation) of five lungs for each treatment. Statistical analysis was performed by using t-test (*p < 0.01)

TABLE 7 Strains used in this work

Strain	Genotype	Source
Afs35	Wild-type strain	FGSC A1159
CEA17	Wild-type strain	D'Enfert et al. (1996)
Ku80	$\DeltaakuB^{ku80}::pyrGAF$	FGSCA1151
Ku80pyrG	$\DeltaakuB^{ku80}::pyrGAF pyrG^-$	da Silva Ferreira et al. (2006)
$\Delta mpkC$	$\Delta mpkC::prtA$	Hagiwara et al. (2014)
$\Delta sakA$	$\Delta sakA::hph$	Hagiwara et al. (2014)
$\Delta mpkC \Delta sakA$	$\Delta mpkC::prtA \Delta sakA::hph$	Bruder Nascimento et al. (2016)
$\Delta atfA^{Afs35}$	$\Delta atfA::prtA$	Hagiwara et al. (2014)
$\Delta atfA^{CEA17}$	$\Delta atfA::pyrGAF pyrG^-$	This work
$\Delta atfB$	$\Delta atfB::pyrGAF pyrG^-$	This work
$\Delta atfC$	$\Delta atfC::pyrGAF pyrG^-$	This work
$\Delta atfD$	$\Delta atfD::pyrGAF pyrG^-$	This work
$\Delta atfA^{Afs35}::atfA^+$	$\Delta atfA::atfA^+::hph$	Hagiwara et al. (2014)
$\Delta atfA^{CEA17}::atfA^+$	$\Delta atfA::atfA^+::prtA^-$	This work
$\Delta atfB::atfB^+$	$\Delta atfB::atfB^+::$	This work
$\Delta atfC::atfC$	$\Delta atfC::atfC^+::$	This work
$\Delta atfD::atfD^+$	$\Delta atfD::atfD^+::$	This work

4.3 | Construction of the *A. fumigatus* mutants

The gene replacement cassettes were constructed by *in vivo* recombination in *S. cerevisiae* as previously described by Colot et al. (2006) and Malavazi and Goldman (2012). Approximately 2.0 kb from the untranslated region 5'-(UTR) and 3'-(UTR) flanking region of the targeted open reading frame regions were selected for primer design. The primers 5F and 3R contained a short homologous sequence to the multiple cloning site of the plasmid pRS426. Both the 5' and 3' UTR fragments were PCR-amplified from *A. fumigatus* genomic DNA (gDNA). The *pyrG* gene, which was placed within the cassette as a prototrophic marker, was amplified from the pCDA21 plasmid. The deletion cassette was generated by transforming each fragment along with the plasmid pRS426 cut with *Bam*HI/*Eco*RI into the *S. cerevisiae* strain SC94721, using the lithium acetate method (Schiestl & Gietz, 1989). The DNA from the transformants was extracted by the method described by Goldman et al. (2003). The cassette was PCR-amplified from these plasmids utilizing TaKaRa Ex Taq™ DNA Polymerase (Clontech Takara Bio) and used for *A. fumigatus* transformation. Southern blot analyses demonstrated that the transformation cassettes had integrated homologously at the targeted loci. The single gene deletion strains were complemented by co-transforming a DNA fragment (approximately 1 kb from each 5' and 3' flanking regions plus the open reading frame) together with the pHATa (Herrera-Estrella, Goldman, & Van Montagu, 1990) and selecting for hygromycin resistance on MM plates containing 250 mg/ml of hygromycin B. All the primer sequences are described in Table S19.

Southern blot and PCR analyses were used to demonstrate that the cassettes had integrated homologously at the targeted *A. fumigatus* loci. Genomic DNA from *A. fumigatus* was extracted by grinding frozen mycelia in liquid nitrogen and then gDNA was extracted as previously described (Malavazi & Goldman, 2012). Standard techniques for manipulation of DNA were carried out as described (Sambrook & Russell, 2001). For Southern blot analysis, restricted chromosomal DNA fragments were separated on 1% agarose gel and blotted onto

Hybond N⁺ nylon membranes (GE Healthcare). Probes were labeled using [α -³²P]dCTP using the Random Primers DNA Labeling System (Life Technologies). Labeled membranes were exposed to X-ray films, which were scanned for image processing. Southern blot and PCR schemes are shown in Figures S6 and S7.

4.4 | Phenotypic assays

The phenotypes of the deletion mutant were evaluated either by radial growth or assessing the initial growth of a droplet of conidia from a serial dilution, at different temperatures, in the presence or absence of oxidative and osmotic stressing agents. Drop out experiments were performed using 5 μ l of a 10-fold dilution series starting at a concentration of 2×10^7 for the wild-type and mutant strains spotted on different media and grown for 48 hr at 37°C.

4.5 | RNA extraction and real-time PCR reactions

Post-treatment, mycelia were harvested by filtration, washed twice with H₂O, and immediately frozen in liquid nitrogen. For total RNA isolation, mycelia were ground in liquid nitrogen. Total RNA was extracted using Trizol (Invitrogen). The integrity of the RNA isolated from each treatment was analyzed using the Agilent 2100 Bioanalyzer system. For real-time PCR experiments, RNase free DNase I treatment was carried out as previously described by Semighini, Marins, Goldman, and Goldman (2002). RNA-seq data (see below) were exploited to select genes for normalization of the quantitative PCR data, applying the method of Yim et al. (2015). Briefly, genes with a coefficient of variation smaller than 20%, behaving in a normally distributed manner, and with large expression values (FPKM) were selected. After experimental validation, gene *asp7/Afu4g06670* was selected. Twenty micrograms of total RNA was treated with DNase, purified using an RNeasy kit (Qiagen), and cDNA was generated using the SuperScript III First Strand Synthesis system (Invitrogen) with oligo(dT) primers, according to manufacturer's instructions. Quantitative real-time PCR was performed for

each primer pair using known amounts of target obtained by serial dilutions of genomic DNA as the template over several orders of magnitude. The initial template amount used in the parallel reaction mixtures was estimated, using 30 Mb as the *A. nidulans* genome size. A linear relationship was obtained by plotting the threshold cycle against the logarithm of known amount of initial template. The equation of the line that best fits the data was determined by regression analysis. The R^2 value was calculated for each dataset to estimate the accuracy of the real-time PCR as a quantification method. The number of target copies contained in an unknown sample was determined by extrapolation from the linear regression of the standard curve obtained for each primer. In all experiments, appropriate negative controls containing no template DNA or RNA were subjected to the same procedure to exclude or detect any possible contamination or carryover. Each sample was repeated at least three times. All reaction mixtures were analyzed by agarose gel electrophoresis to confirm that only one PCR product was synthesized. The results were normalized using the C_T s obtained for the gene *aspf7/Afu4g06670* RNA amplifications run on the same plate. All the PCR reactions were performed using an ABI 7500 Fast Real-Time PCR System (Applied Biosystems) and SYBR® Green PCR Master Mix (Applied Biosystems). The primer sets for the analyses are listed in Table S11.

4.6 | *Galleria mellonella* experiments and the murine model of pulmonary aspergillosis and fungal burden

Galleria mellonella larvae were obtained by breeding adult moths (Fuchs, O'Brien, Khoury, & Mylonakis, 2010). *G. mellonella* larvae of a similar size were selected (approximately 275–330 mg) and kept without food in glass container (Petri dishes), at 37°C, in darkness for 24 hr prior to use. *A. fumigatus* conidia were obtained by growing on YAG media culture for 2 days. The conidia were harvested in PBS and filtered through a Miracloth (Calbiochem). The concentration of conidia was estimated by using hemocytometer and resuspended at a concentration of 2.0×10^8 conidia/ml. The viability of the conidia was determined by incubating on YAG media culture, at 37°C, for 48 hr. Inoculum ($5 \mu\text{l}$ of a 2×10^8 conidia/ml) from the wild-type, mutant, and complemented strains (1×10^6 conidia/larva) were used to investigate the virulence of *A. fumigatus* against *G. mellonella*. Ten *G. mellonella* in the final (sixth) instar larval stage of development were used per condition in all assays. The control group was the larvae inoculated with $5 \mu\text{l}$ of PBS to observe the killing due to physical trauma. The inoculum was performed by using Hamilton syringe (7000.5KH) and $5 \mu\text{l}$ into the haemocoel of each larva via the last left proleg. After, the larvae were incubated in a glass container (Petri dishes) at 37°C in the dark. The larval killing was scored daily. Larvae were considered dead by presenting the absence of movement in response to touch.

Outbred female mice (BALB/c strain; body weight, 20 to 22 g) were housed in vented cages containing five animals. Mice were immunosuppressed with cyclophosphamide (150 mg/kg of body weight), which was administered intraperitoneally on days -4, -1, and 2 prior to and post-infection. Hydrocortisonacetate (200 mg/kg of body weight) was injected subcutaneously on day -3. *A. fumigatus* strains were grown on YAG for 2 days prior to infection. Fresh conidia were harvested in PBS and filtered through a Miracloth (Calbiochem). Conidial suspensions were spun for 5 min at $3,000 \times g$, washed three

times with PBS, counted using a hemocytometer, and resuspended at a concentration of 5.0×10^6 conidia/ml. The viability of the administered inoculum was determined by incubating a serial dilution of the conidia on YAG medium, at 37°C. Mice were anesthetized by halothane inhalation and infected by intranasal instillation of 1.0×10^5 conidia in $20 \mu\text{l}$ of PBS. As a negative control, a group of five mice received PBS only. Mice were weighed every 24 hr from the day of infection and visually inspected twice daily. The statistical significance of the comparative survival values was calculated using log rank analysis and the Prism statistical analysis package.

To investigate fungal burden in murine lungs, mice were immunosuppressed with cyclophosphamide (150 mg/kg of body weight), which was administered intraperitoneally on days -4 and -1, while hydrocortisonacetate was injected subcutaneously (200 mg/kg) on day -3. Five mice per group were intranasally inoculated with $20 \mu\text{l}$ of a 1×10^6 conidial suspension. A higher inoculum, in comparison with the survival experiments, was used to increase fungal DNA detection. Animals were sacrificed 72 hr post-infection, and the lungs were harvested and immediately frozen in liquid nitrogen. Samples were homogenized by vortexing with glass beads for 10 min, and DNA was extracted via the phenol-chloroform method. DNA quantity and quality were assessed using a NanoDrop 2000 spectrophotometer (Thermo Scientific). At least 500 μg of total DNA from each sample was used for quantitative real-time PCRs. PCR reactions were performed using an ABI 7500 Fast Real-Time PCR System (Applied Biosystems, USA) and SYBR Green detection. SYBR® Green PCR Master Mix (Applied Biosystems, USA) was used for reaction mixture preparation. The primer sets for the analyses were used to amplify the 18S rRNA region of *A. fumigatus* (18S rRNA *Afu* sybr FW, 5'-GACCTCGGCCCTTAAATAGC-3' and 18S rRNA *Afu* sybr Rv, 5'-CTCGGCCAAGGTGATGTACT-3') and an intronic region of mouse GAPDH, encoding glyceraldehyde-3-phosphate dehydrogenase (GAPDH mouse sybr FW, 5'-GAGGGACTTGGAGGACACAG-3' and GAPDH mouse sybr Rv, 5'-ACATCACCCCCATCACTCAT-3'). Six-point standard curves were calculated using serial dilutions of gDNA from all the *A. fumigatus* strains used and the uninfected mouse lung. Fungal and mouse DNA quantities were obtained from the threshold cycle values from an appropriate standard curve. Fungal burden was determined as the ratio between picograms of fungal and micrograms of mouse DNA.

4.7 | RNA sequencing

Aspergillus fumigatus conidia (1×10^6 sp/ml) from the strains Afs35, ΔsakA , ΔmpkC , and $\Delta\text{mpkC} \Delta\text{sakA}$ were inoculated into liquid YPD (1% yeast extract, 1% polypeptone, and 1% glucose) and cultured, in three biological replicates, for 16 hr prior to addition of one half volume of 3 M sorbitol (final concentration: 1 M). Mycelia were harvested and frozen in liquid nitrogen before and 10 min after the addition of sorbitol. For total RNA isolation, mycelia were ground in liquid nitrogen. Total RNA was extracted using Trizol (Invitrogen), treated with RNase-free DNase I (Fermentas) and purified using an RNeasy Kit (Qiagen) according to manufacturer's instructions. The RNA from each treatment was quantified using a NanoDrop and Qubit fluorometer and analyzed using an Agilent 2100 Bioanalyzer system to assess the

integrity of the RNA. RNA integrity number was calculated; the RNA sample had an RNA integrity number of 9.0–9.5.

An Illumina TruSeq Stranded mRNA Sample Preparation kit was used. Briefly, polyA-containing mRNA molecules were selected using polyT oligo-attached magnetic beads. Fragmentation and paired-end library preparation were done using divalent cations and thermal fragmentation. First-strand cDNA synthesis was performed using reverse transcriptase (Superscript II) and random primers. This was followed by second-strand cDNA synthesis using DNA polymerase I and RNase H and dUTP in place of dTTP. AMPure XP beads were used to separate the dsDNA from the second strand. At the end of this process, we had blunt-ended cDNA to which a single "A" nucleotide was added at the 3' end to prevent them from ligating to one another during the adapter ligation reaction. A corresponding single "T" nucleotide on the 3' end of the adapter provided a complementary overhang for ligating the adapter to the fragment. The products were then purified and enriched using a PCR to selectively enrich those DNA fragments that have adapter molecules on both ends and to amplify the amount of DNA in the library. The PCR was performed with a PCR Primer Cocktail from the Illumina kit that anneals to the ends of the adapters. Libraries were sequenced (2×100 bp) on a HiSeq 2500 instrument, sequencing approximately 17.3×10^6 fragments per sample. Short reads were submitted to the NCBI's Short Read Archive under the Bioproject: PRJNA327227.

Obtained fastq files were quality checked with FastQC (<http://www.bioinformatics.babraham.ac.uk/projects/fastqc/>) and cleaned (quality trim, adaptor removal, and minimum length filtering) with Trimmomatic (Bolger, Lohse, & Usadel, 2014); finally, ribosomal RNA was removed using SortMeRNA (Kopylova, Noé, & Touzet, 2012). High-quality reads were mapped to the *A. fumigatus* Af293 genome sequence (Nierman et al., 2005) using Tophat2 (Kim et al., 2013) in strand-specific mode. Saturation of sequencing effort was assessed by counting the number of detected exon–exon junctions at different subsampling levels of the total high-quality reads, using RSeQC (Wang, Wang, & Li, 2012). All samples achieved saturation of known exon–exon junctions. Reproducibility among biological replicates was assessed by exploring a multidimensional scaling plot of the top 500 genes that have the largest biological variation between the libraries and by pair-wise measuring the Pearson correlation among the replicates over the whole set of genes, which lead to the removal of four samples from downstream analyses ($\Delta mpkC$ at 10 min replicate 1, $\Delta mpkC$ at 0 min replicate 1, $\Delta mpkC \Delta sakA$ at 10 min replicate 3, and $\Delta mpkC \Delta sakA$ at 0 min replicate 3). In order to assess transcript abundance, exonic reads were counted in a strand-specific way using the Rsubread library (Liao, Smyth, & Shi, 2013) from the Bioconductor suite (Huber et al., 2015). Calling of differentially expressed genes was carried out in EdgeR (Robinson, McCarthy, & Smyth, 2010), controlling for an FDR of 0.05 (Benjamini, Drai, Elmer, Kafkafi, & Golani, 2001).

4.8 | Bioinformatics analysis of the HOG1/SakA/MpkC function in *S. cerevisiae*, *C. albicans*, *S. pombe*, and *A. fumigatus*

We have used the dataset from Enjalbert et al. (2006) describing the genes induced by osmotic stress in *S. cerevisiae*, *S. pombe*, and

C. albicans. We selected *A. fumigatus* orthologues by using reciprocal Blastp analysis with a cutoff *e*-value of $10e-5$, where the best bidirectional hits were selected. We compared the expression of all these four datasets by following the same definition provided by Enjalbert et al. (2006) for Hog1-regulated genes: 50% higher in HOG1 cells than in hog1 cells ($HOG1/hog1 \geq 1.5$) or at least 40% lower in HOG1 cells than in hog1 cells ($HOG1/hog1 \leq 0.60$). GO enrichment analyses of the significantly modulated gene sets were performed in *A. fumigatus* by using FetGOat (Nitsche et al., 2011). Gene annotation and GO enrichment for *S. cerevisiae*, *S. pombe*, and *C. albicans* were obtained from SGD (<http://www.yeastgenome.org/>), CGD (<http://www.candidagenome.org/>), and PomBase (<http://www.pombase.org/>), respectively, with a critical FDR *p*-value of 0.05, a minimal annotation group size of 2, and the Benjamini and Hochberg correction. We have removed the functionally redundant categories and weak probability groups with no clear significance in the response to make simpler the list of functional categories.

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3 **Capítulo 3.2:** Genetic and protein interactions of the *Aspergillus fumigatus* basic
4 leucine zipper (bZIP) transcription factors AtfA, AtfB, AtfC, and AtfD are important
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**Genetic and protein interactions of the *Aspergillus fumigatus* basic
leucine zipper (bZIP) transcription factors AtfA, AtfB, AtfC, and AtfD are
important to cope with different stress conditions**

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43

44 **Abstract**

45 *Aspergillus fumigatus* is an opportunistic fungus, capable of causing Invasive
46 Aspergillosis (IA) in immunocompromised patients, recently transplanted or
47 undergoing chemotherapy. The biggest challenges in fighting IA is the ability of
48 the fungus to adapt and resist to different stressing conditions. This is because,
49 the fungus has a highly efficient biochemical machinery, activating mediators
50 such as kinases, which in turn recruit transcription factors for the modulation of
51 genetic transcription to occur. We have demonstrated that the transcription
52 factors AtfA, -B, -C and -D respond quickly and robustly to osmotic, oxidative and
53 cell wall stresses. These TFs are activated by interactions with SakA^{Hog1p}, after
54 the fungal cells identify external stresses. In addition, these TFs are able to form
55 heterodimers, increasing the range of their ability to bind to different DNA regions.
56 In addition, we demonstrate that TFs are involved in the accumulation of
57 trehalose, glycogen and metabolic assimilation of different carbon sources. Our
58 results suggest that AtfA-D respond not only to the MAPK HOG pathway, but also
59 to the cell wall integrity (CWI) signaling pathways.

60

61 **Keywords:** *Aspergillus fumigatus*, transcription factors, Mitogen-activated protein
62 kinase (MAPK), High Osmolarity Glycerol (HOG), Cell Wall Integrity (CWI).

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Introduction

Aspergillus fumigatus, is a filamentous saprophytic fungus, which is commonly found in the environment (Prasad et al., 2016). This fungus produces conidia, which facilitates its dispersion in the air, and due to its small size (2-3µm) and its abundance in the environment, humans inhale daily hundreds of these conidia, which can lodge in their lower respiratory tract (Tischler and Hohl, 2019) and cause the disease called Invasive Pulmonary Aspergillosis (AI) (Prasad et al., 2016). The mortality rate of infection caused by *A. fumigatus* can reach 85% in immunocompromised individuals or those who have undergone transplantation and/or chemotherapy procedures. In these patients, the lines of defense, such as coordinated action of the respiratory epithelium, resident macrophages in the lungs, neutrophils and monocytes, recruited by the host to eliminate conidia do not work efficiently (van de Veerdonk et al., 2017; Tischler and Hohl, 2019). The virulence of the pathogen is multifactorial, where several phenotypes can influence the establishment of the disease. It is known that different signaling pathways used by *A. fumigatus* are responsible for modulating the biochemical machinery of the pathogen, thus favoring rapid adaptation to different environmental stresses and the host immune system (Silva et al., 2017). The highly conserved mitogen-activated protein kinase (MAPK) is one of these signaling pathways (Pearson et al., 2001; Risipail et al., 2009; Day and Quinn, 2019). MAPKs are important for relaying, integrating, and amplifying intracellular signals involved in many cellular processes (Pearson et al., 2001; Risipail et al., 2009). *A. fumigatus* has four MAPK cascades (i) MpkA, a central regulator of the cell wall integrity pathway (CWI) (VALIANTE, 2015); (ii) MpkB, involved in the regulation of the production of secondary metabolites, in particular DHN-melanin (Manfiolli et al., 2019; da Costa Filho et al., 2020); and (iii) MpkC and SakA, orthologues of *S. cerevisiae* Hog1p, the main regulator of the HOG-MAPK pathway (Risipail, 2009; Silva et al., 2017).

The central signal transduction pathway operating during hyperosmotic and oxidative stresses is the High Osmolarity Glycerol (HOG) MAPK cascade (Martínez-Montañés et al., 2010; de Nadal and Posas, 2015). In yeast, the HOG

pathway has signaling branches controlled by the osmosensors Sln1p and Sho1p, with independently function (Hohmamm, 2002). Under stressful conditions Sln1p phosphorylates Ypd1p which transfers its phosphate to the Ssk1p regulator, initiating the kinases phosphorylation of the HOG pathway. Another branch comprises the osmosensor Sho1p, putative osmosensors Hkr1p, Msb2p and the membrane anchor protein Opy2p, responsible for initiating the process of activating the cascade of kinases Ste11p, Pbs2p and Hog1p (Hohmamm, 2002). Recently, we have shown that some elements of these pathways are conserved in *A. fumigatus* activating not only SakA homologue but also MpkA, a MAPK from the CWI (Silva et al., 2020; Manfiolli et al., 2019). Upon different kinds of stress *A. nidulans* SakA and *A. fumigatus* SakA and MpkC are translocated to the nucleus (Lara-Rojas et al., 2011; Bruder Nascimento et al., 2016). *A. nidulans* SakA accumulates in the nucleus and physically interacts with the basic leucine zipper transcription factor (TF b-ZIP) AtfA^{Atf1p}, which activates catalase genes during oxidative stress (Lara-Rojas et al., 2011). This TF, the mammalian ATF2 homologue, binds to CRE regions (T [G/T] ACGT [C A] A) of target genes in response to stressful conditions (Hagiwara et al., 2008; Sakamoto et al., 2009). In *A. fumigatus* AtfA homologue was first described as dependent on the stress-activated kinase SakA and the cyclic AMP-protein kinase A (cAMP-PKA) pathways and important for the expression of DprA dehydrin, a protein important to cope with oxidative stress conditions and phagocytic killing (Wong Sak et al., 2011). *A. fumigatus* *atfA* null mutant was shown to be important for long-term viability of conidia, with significant sensitivity to high temperature and oxidative stress, and reduced conidial trehalose content (Hagiwara et al., 2014). Transcriptomic analysis comparing hyphae, resting conidia, and 1-h grown conidia in *A. fumigatus*, *A. niger*, and *A. oryzae*. Showed that AtfA is important for the regulation of conidial stress-related and germination genes, suggesting a major role for AtfA in *Aspergillus* conidial dormancy (Hagiwara et al., 2016). These authors also identified three putative AtfA homologues, AtfB, -C, and -D that have a relatively conserved bZIP-type DNA-binding domain (Hagiwara et al., 2014). We have characterized null mutants for all these four *A. fumigatus* b-ZIP TFs. These four TFs have their expression dependent on $\Delta sakA$ and $\Delta mpkC$ $\Delta sakA$ post-osmotic stress, and displayed several phenotypes related to osmotic, oxidative, and cell wall stresses (Silva et al., 2017). The $\Delta atfA$ and $\Delta atfB$ were

shown to be avirulent and to have attenuated virulence, respectively, in both *Galleria mellonella* and a neutropenic murine model of invasive pulmonary aspergillosis (Silva et al., 2017).

TFs have the potential to elucidate virulence mechanisms and provide information that can help us highlight new targets for antifungal drugs. TFs, abundantly regulate several important genes for a given biological response (Bultman et al., 2017). In addition, b-ZIP TFs have the ability to form homo- or heterodimers, a paradigm that provides combinatorial control of gene expression in eukaryotes, increasing the complexity of the genetic regulatory network and allowing the emergence of new control circuits (Klemm et al., 1998; Pogenberg et al., 2014). In the present work, we continued the investigation on *A. fumigatus* AtfA-D TFs characterizing possible genetic and physical interactions between them after stressing conditions. Here, we showed that these four TFs are not only interacting at genetic level but also physically forming all the possible combinations of heterodimers. We also identified that AtfA-D physically interact with the MAPK SakA upon osmotic and cell wall stresses.

Materials and Methods

Strains and culture media

The *A. fumigatus* strains used during the study are listed in the **Supplementary Table S1**. The culture used were mainly complete and/or minimal media. The complete medium consists of, YAG (2% w/v dextrose, 0.5% w/v yeast extract, 0.1% v/v element trace solution, 2% w/v agar), YUU (Medium YAG plus uridine and uracil (1.2 g/L), and YG or YG + UU media with the same composition, but without agar. The minimum medium (MM) consists of 1% glucose, 0.1 element trace solution % v/v, 20X salt solutions, pH 6.5 with or without the addition of 2% (w/v) agar (Kafer, 1997). The strains were grown at 37°C for 5 days.

Construction of mutant strains

All sequences are in accordance with the AspGD database (<http://www.aspgd.org/>). The deletion cassette was constructed by *in vivo*

recombination in *S. cerevisiae* as described (Colot et al., 2006). About 1.0 kb of the flanking region of the 5'-UTR and 3'-UTR regions of the target ORF regions were selected for primer design. The 5'Fw and 3Rv primers contained a short sequence homologous to plasmid pRS426 and a checkmark *pyrG* or pPTRA. Both 5' and 3'-UTR fragments were amplified by PCR from *A. fumigatus* genomic DNA (gDNA). The *pyrG* gene placed inside the cassette as a prototrophic marker was amplified from the vector pCDA21. The deletion cassette was generated by transformation into *S. cerevisiae* SC94721, using the lithium acetate method (Schiestl & Gietz, 1989), plus the fragments, together with the plasmid pRS426 digested in two sites with the enzyme *BamHI* and *EcoRI* (New England Biolabs UK). The DNA of the transformants was extracted using the method described (Goldman et al., 2003). The deletion cassette was amplified by PCR from these plasmids using TaKaRa Ex Taq™ DNA polymerase (Clontech Takara Bio) and used for the transformation of *A. fumigatus*. The same protocol was applied to the construction of the double mutants. However, the check mark used for the gene deletion was amplified from the plasmid pPTRI. Colonies of null mutants were selected by purifying colonies in MM with 1 µg / ml pyrimiamine hydrobromide (Sigma). Southern blot analysis demonstrated that the transformation cassette was homologous. Deletion strains of a single gene were complemented by co-transformation, approximately 1.0kb of the 5' and 3'-UTR plus ORF together with the plasmid pPTRI according to (Herrera-Estrella et al., 1990) with modifications. Complements were selected by purifying colonies in MM with 1ug / ml of piritiamine hydrobromide.

The homologous and / or heterologous recombination to gene replacement was confirmed by PCR and Southern blot. The absence of mRNA from the target genes was verified by qPCR. All primers used for the constructions are listed in **Supplementary Table S2**.

Characterization of mutant null strains

The characterization of sensitivity and / or resistance was evaluated by radial growth comparing mutant and wild strains. 5ul of a 2×10^7 suspension were dropped into MM with or without stressors, the plates were cultured for 5 days at

37°C. After that period, the radial diameter was measured and the values were used for statistical analysis. The different stressful conditions were ions a with the addition of calcofluor white (CFW), Congo Red (CR), menadione, sorbitol and caspofungin.

Growth of strains in alternative carbon source

To assess growth in different carbon sources, we use modified MM. Sodium acetate (1%) or ethanol (1%) was added to the MM instead of glucose (1%) (Kafer, 1997). The strains were grown for 5 days at 37°C.

Statistical analysis

Data were analyzed using "Prism, GraphPad)" Two-way ANOVA "followed by" Bonferroni post-tests ". Significance levels were * $p < 0.1$; ** $p < 0.01$ and * ** $p < 0.001$. All the strains were compared with the single mutants and the Wild-type.

Protein extraction and Western blot

The strains were inoculated (1×10^7 conidia) in 250-ml erlenmeyer's flasks with 50 ml of YG medium and grown in a rotatory shaker (200 rpm) at 37°C for 16 h before being exposed to different stressing agents, such as Congo Red, sorbitol. The total protein extracts were obtained through mycelia ground with The macerated mycelia were resuspended (500mg) in 1 ml of B250 lysis buffer (250 mM NaCl, 100 mM Tris-HCl pH 7.5, 10% glycerol, 1Mm EDTA and 0.1% NP-40) supplemented with 1.5 ml/L of 1M DTT, 100mL of complete protease inhibitor Cocktail without EDTA (Roche), 3 ml/L of 0.5M Benzamidine, 10 ml/L of 100X phosphatase inhibitors (1M NaF, 5M sodium orthovanadate , 8M glycerol phosphate) and 10mL/L of 100 mM PMSF.). The samples were centrifuged at 13,000g for 10 min at 4°C. The supernatants were removed and quantified by Bradford assay (BioRad). Western blots were performed as previously described (de Assis et al., 2018).

GFP-Trap and anti-HA (co-IP)

To perform the co-immunoprecipitation (co-IP) assays, AtfA-D:GFP strains had SakA marked with 3xHA at the C-terminus (SakA:3xHA). The strains were grown for 16 h in liquid YG and were further exposed to two conditions, these being, sorbitol 1.2 M (10 min) and CR 300ug / ml (10 min). The co-IP experiments were carried out with GFP-Trap (Chromotek) according to (Manfiolli et al., 2017). To perform co-IP tests, the mycelia were frozen with liquid nitrogen and macerated with the aid of a crucible and pestle. The samples were normalized and the same amount of protein was added to 20µL of GFP-trap, the resin was washed three times with resuspension buffer before incubation. The protein extracts and the resin were then incubated with shaking at 4°C for 4 hours. After incubation, the resin was washed three times in B250 buffer. To release proteins from the resin, the samples were incubated with sample buffer and boiled at 98°C for 5 min. The proteins were transferred from a 10% SDS-PAGE gel to a nitrocellulose membrane for a Western blot assay using a Trans-Blot turbo transfer system (Bio-Rad). GFP-labeled AtfA-D were detected using a rabbit anti-GFP antibody (Cell Signaling Technology) at a dilution of 1:1,000. For the detection of HA-labeled proteins, a mouse anti-HA monoclonal antibody (Sigma) was used in the 1:2,000 dilution. Both primary antibodies were identified with the conjugated anti-mouse IgG antibody (Cell Signaling Technology) used at 1:1,000 dilution as a secondary antibody.

Measurement of glucose in the supernatant

The strains were inoculated (1×10^7 conidia) in 250ml erlenmeyer's flasks with 50 ml of YG medium and grown in a rotatory shaker (200 rpm) at 37°C for 24h. After that period, the mycelia were then transferred to minimum medium plus 1% glucose as a carbon source for another 24 hours. The amount of free glucose remaining in the medium was measured using the GOD-PAP Glucose Liquid Stable Monoreagent kit (Laboratories LaborLab Ltd. Guarulhos, São Paulo, Brazil), according to the manufacturer's instructions. Glucose uptake was calculated by determining the difference in glucose present in the initial medium and after 24 hours of incubation.

258

259 **Mycelia dry weight**

260 The strains were inoculated (1×10^7 conidia) in 250ml erlenmeyer's flasks with 50
261 ml of YG medium and grown in a rotatory shaker (200 rpm) at 37°C for 24h. After
262 that period, the mycelia were transferred to minimum medium plus 1% glucose
263 as a carbon source for 24 hours. Then the mycelia were filtered and taken to the
264 lyophilizate for 48 hours. The dry weight of each strain was compared to the dry
265 weight of the wild type mycelium to normalization.

266

267 **Intracellular glycogen and trehalose concentrations**

268 Intracellular glycogen and trehalose levels were measured in mycelia grown 24h
269 in MM supplemented with glucose as a carbon source as previously described
270 (de Assis et al, 2019).

271

272 **Virulence analysis in *Galleria mellonella* models**

273 *Galleria mellonella* larvae were obtained by reproducing adult larvae (Fuchs et
274 al, 2010). The larvae used for the infection were in the last larval stage of
275 development (sixth week). All selected larvae weighed approximately 300mg,
276 and were restricted to food for 24 hours before the experiment. The fresh conidia
277 of each *A. fumigatus* strain were counted using a hemocytometer. The initial
278 concentration of conidial suspensions for infections were 2×10^8 conidia/ml, total
279 of 5 μ l (1×10^6 conidia/ larvae) of each suspension was inoculated by larva. The
280 control group was composed of larvae whose 5 μ l of PBS were injected to
281 observe death from physical trauma. The inoculum was carried out with the
282 Hamilton syringe (7000.5 KH) through the last left ear. After infection, the larvae
283 were kept with food restrictions, at 37°C in Petri dishes in the dark, and scored
284 daily. The larvae were considered dead due to lack of movement in response to
285 touch. The viability of the inoculum administered was determined by serial dilution
286 of the conidia in YAG medium and incubating the plates at 37°C for 72h.

287

Results

Double mutants for the TFs AtfA-D display increased sensitivity to osmotic, oxidative and cell wall stresses in *A. fumigatus*. In a previous publication by our research group (Silva et al., 2016), we showed that the TFs AtfA-D are modulated by MAP kinases SakA and MpkC and function downstream the HOG pathway and therefore regulate genes involved in the adaptation of *A. fumigatus* to stressful conditions. These four TFs belong to a small bZIP family whose proteins are between 185 to 555 aa (**Figure 1A**). Multiple alignment of the bZIP domain of AtfA has about 58 to 65 % identity with AtfB-D. The bZip domains were identified by SMART (<http://smart.embl-heidelberg.de>) and **Figure 1A** shows the linear protein structures with the correspondent bZip domain position. Protein modeling predictions based on structural templates allowed to observe the AtfA-D structural configuration (**Figure 1B**) and the binding site deduced region (**Figure 1C**). To further characterize these TFs, we constructed double deletions mutants for these four null TF genes, therefore generating six double mutants ($\Delta atfA \Delta atfB$, $\Delta atfA \Delta atfC$, $\Delta atfA \Delta atfD$, $\Delta atfB \Delta atfC$, $\Delta atfB \Delta atfD$, and $\Delta atfC \Delta atfD$) and evaluated the possible genetic interactions among the mutants in the presence of different stressing conditions.

Growth of the double deletion mutants in minimum medium (MM) for 5 days at 37 °C provided initial evidence of the differences and synergism between single TF mutants (**Figures 1D-E**). The mutant $\Delta atfA$ displayed reduced growth when compared to the wild-type and the other single deletion mutants (**Figures 1D-E**). Deleting either *atfB* or *atfD* in the $\Delta atfA$ background restored wild-type phenotype suggesting these two mutations can suppress the $\Delta atfA$ growth defect (**Figures 1D-E**); however, deleting *atfC* in the same background mimicked the $\Delta atfA$ phenotype (**Figures 1D-E**). Reduced growth was also observed for the $\Delta atfB \Delta atfC$ mutant (**Figures 1D-E**). Interestingly, deleting *atfD* in both $\Delta atfA$ and $\Delta atfB$ backgrounds resulted in sectorized growth, what was not observed for $\Delta atfC \Delta atfD$ (**Figures 1F**). Table 1 shows the different genetic interactions between the mutants.

Figure 1- *A. fumigatus* protein structure of AtfA-D family. **(A)** Protein sequence AtfA (Afu3g11330), length 555 aminoacids (aa), conserved bZip domain sequence between aa 441 to 505; AtfB (Afu5g12960), length 328 aa, conserved bZip domain sequence between aa 161 to 225; AtfC (Afu1g17360), length 286 aa, conserved bZip domain sequence between aa 139 to 203; AtfD (Afu6g12150), length 185 aa, conserved bZip domain sequence between aa 100 to 164aa. **(B)** Modeling prediction of protein structure (C-score/TM-score: AtfA - 0.55/0.64 $\pm$ 0.13, AtfB -2.57/0.42 $\pm$ 0.14, AtfC -2.78/0.40 $\pm$ 0.13, AtfD - 2.10/0.46 $\pm$ 0.15. **(C)** Deduction of binding site position by structural comparison (PDB Hit/C-score: AtfA 2h7hB/0.11, AtfB 4evpA/0.11, AtfC 3j47U/0.15, AtfD 3hd7A/0.18. **(D-E)**. Growth phenotypes of the wild-type, $\Delta atfA$ $\Delta atfB$, $\Delta atfA$ $\Delta atfC$, $\Delta atfA$ $\Delta atfD$, $\Delta atfB$ $\Delta atfC$, $\Delta atfB$ $\Delta atfD$ and $\Delta atfC$ $\Delta atfD$ strains. The strains were grown for 5 days at 37 °C on MM.

333

334

335 In the presence of CFW, the double $\Delta atfA \Delta atfB$ is as sensitive as $\Delta atfA$ and $\Delta atfB$
 336 to CFW (90 μ g/ml) while $\Delta atfA \Delta atfC$ recapitulates $\Delta atfA$ growth (**Figure 2A**). The
 337 $\Delta atfB \Delta atfC$ mutant is more sensitive to CFW (90 μ g/ml) than the corresponding
 338 single mutants, suggesting additive interaction between these two mutants
 339 (**Figure 2A, Table 1**). Deleting *atfD* in the $\Delta atfA$ background restored wild-type
 340 phenotype suggesting this mutation can suppress the $\Delta atfA$ growth defect
 341 (**Figure 2A**). The $\Delta atfB \Delta atfD$ and $\Delta atfC \Delta atfD$ mutants are as sensitive as the
 342 single mutants, suggesting the single mutants are not interacting (**Figure 2A and**
 343 **Table 1**). In the presence of CR (50 μ g/ml), all the double mutants behave like
 344 the single mutants, except for $\Delta atfA \Delta atfC$ and $\Delta atfB \Delta atfC$ that are more
 345 sensitive than the corresponding single mutants, suggesting additive interaction

(**Figure 2B** and **Table 1**). The double mutants $\Delta atfB \Delta atfC$, $\Delta atfB \Delta atfD$ and $\Delta atfC \Delta atfD$ are more resistant to higher osmotic concentrations (1.2 M sorbitol) mimicking the single mutant phenotypes of $\Delta atfC$ and $\Delta atfD$ (**Figure 2C** and **Table 1**). In contrast, deletion of *atfA* in $\Delta atfC$ and $\Delta atfD$ background increase the sensitivity to 1.2 M sorbitol, suggesting a suppression phenotype (**Figure 2C** and **Table 1**).

We also investigated the response to two agents that cause oxidative stress, H_2O_2 and menadione (**Figures 2E** and **2F**). Growth in different H_2O_2 concentrations showed that $\Delta atfA \Delta atfC$ and $\Delta atfA \Delta atfD$ recapitulate the $\Delta atfA$ phenotype, suggesting there are no genetic interactions between these mutants (**Figure 2E** and **Table 1**). In contrast deleting *atfB* in the $\Delta atfA$ background partially suppresses the H_2O_2 sensitivity while $\Delta atfB$ in the $\Delta atfC$ or $\Delta atfD$ background increases H_2O_2 sensitivity (**Figure 2E** and **Table 1**). In the presence of 0.05 mM menadione, the double mutants $\Delta atfA \Delta atfB$, $\Delta atfA \Delta atfC$, $\Delta atfB \Delta atfC$ and $\Delta atfA \Delta atfD$ are as sensitive as the corresponding single mutants (**Figure 2F** and **Table 1**). In contrast the deletion of *atfD* in the $\Delta atfA$ or $\Delta atfD$ background increases the H_2O_2 sensitivity (**Figure 2F** and **Table 1**).

Taken together, the data indicates complex genetic interactions among these TFs that could impact the response to different kinds of stressful conditions.

Figure 2- *A. fumigatus* double null mutants indicate genetic interactions among $\Delta atfA$, $\Delta atfB$, $\Delta atfC$ and $\Delta atfD$. Growth phenotypes of the wild-type, $\Delta atfA \Delta atfB$, $\Delta atfA \Delta atfC$, $\Delta atfA \Delta atfD$, $\Delta atfB \Delta atfC$, $\Delta atfB \Delta atfD$ and $\Delta atfC \Delta atfD$ strains. **(A)** MM+Calcofluor White (CFW). **(B)** MM+Congo Red (CR). **(I)**, MM+sorbitol. **(C)** Liquid MM+ H_2O_2 . **(D)** MM+menadione, and had their radial growth quantified. The results are the means of measuring the diameter of 3 colonies for each strain $\pm$ standard deviation. The data were analyzed using (Prism, GraphPad) "Two-way ANOVA" followed by "Bonferroni posttests". The levels of significance were of * $p < 0.1$; ** $p < 0.01$ and *** $p < 0.001$ compared to the wild-type strain.

Table 1 – Summary of the genetic interactions between *atfA*, *atfB*, *atfC* e *atfD* double mutants grown on different stressing conditions.

Strains/Phenotypes*	$\Delta atfA \Delta atfB$	$\Delta atfA \Delta atfC$	$\Delta atfA \Delta atfD$	$\Delta atfB \Delta atfC$	$\Delta atfB \Delta atfD$	$\Delta atfC \Delta atfD$
CFW						
CR						
Sorbitol						
H ₂ O ₂						
Menadione						
MM+glucose						
MM+acetate						
MM+ethanol						
Trehalose						
Glycogen						
Virulence						

epistatic interaction
additive interaction
suppression interaction
no interaction

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*MM= Minimal media 1% glucose; CR=Congo Red (50 µg/ml); CFW=Calcofluor White (90 µg/ml); CaCl₂ (500 mM); Sorbitol 1.2 M; Menadione 0.05 M; MM+glucose 1 %; MM+acetate 1 %; MM+ethanol 1 %; and virulence in *Galleria mellonella*

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A. fumigatus AtfA-D:GFP nuclear localization. Because the cell localization of TFs AtfA-D in *A. fumigatus* has not been previously described we investigate the nuclear localization of these TFs in functional AtfA-D:GFP strains (**Figure 3A** and **Supplementary Figure S1**). AtfA:GFP is approximately 97% located in the nucleus of *A. fumigatus*, independently of exposure or not to stressing conditions (**Figures 3A to 3D**). In contrast, about 20 to 30% of AtfB, -C, and -D:GFP locates into the nucleus (**Figures 3A to 3D**). However, under stressing conditions, these TFs tend to translocate to the nucleus. AtfB:GFP translocates to the nucleus about 60 to 80 % upon the addition of sorbitol, H₂O₂, or caspofungin (**Figures 3A to 3D**). AtfC:GFP translocates about 65 to 80 % upon addition of sorbitol and H₂O₂ but only 10 % in the presence of caspofungin (**Figures 3A to 3D**). Finally, AtfD:GFP translocates 30 to 60 in the presence of sorbitol and H₂O₂, respectively, but 60 % in the presence of caspofungin (**Figures 3A to 3D**).

These data suggest AtfA-D are possibly important for the transcriptional regulation of genes involved in the response to osmotic, oxidative, and cell wall stresses.

Figure 3 – AtfA-D:GFP nuclear localization. **(A)** Cellular localization (%) of the *A. fumigatus* AtfA-D:GFP in liquid MM. **(B)** Percentage of cellular localization of AtfA-D:GFP proteins after treatment with caspofungin (0.125 ug / ml). **(C)** Percentage of cellular localization of AtfA-D: GFP proteins after treatment with H₂O₂ 2 mM. **(D)** Percentage of cellular localization of AtfA-D: GFP proteins after treatment with sorbitol 1.0 M. The AtfA-D:GFP strains were grown in liquid MM for 16h at 30°C, after that the treatments with stressors were performed.

The TFs AtfA-D contribute to the adaptation of *A. fumigatus* to different carbon sources. To verify if AtfA-D could influence carbon source utilization we checked the glucose consumption rate in liquid medium for single and double deletion mutants. Single mutants show slower glucose consumption when

compared to the wild-type (**Figures 4A-D**). While the wild-type and $\Delta atfD$ exhaust all glucose in 24 h, the $\Delta atfA$ and $\Delta atfB$ strains consumed only 80% of glucose and $\Delta atfC$ consumed 90%. The $\Delta atfA \Delta atfB$, $\Delta atfA \Delta atfC$, $\Delta atfB \Delta atfC$ mimicked the phenotypes of either $\Delta atfA$, $\Delta atfB$ or $\Delta atfC$ (**Figures 4E, 4F, 4H, and Table 1**) while deletion of either *atfA*, *atfB* or *atfC* in the $\Delta atfD$ recapitulates the $\Delta atfD$ phenotype (**Figures 4G, 4I, 4J, and Table 1**). We measure the dry weight of mycelia to evaluate if a reduced glucose consumption would affect the growth of strains (**Supplementary Figure S2**). The single mutant $\Delta atfA$ shows a 50 % reduction in dry weight when compared to the wild-type (**Supplementary Figure S2A**), while the double mutants $\Delta atfA \Delta atfC$, $\Delta atfA \Delta atfD$, $\Delta atfB \Delta atfC$, and $\Delta atfB \Delta atfD$ show 40 to 50 % dry weight reduction when compared to the wild-type strain (**Supplementary Figure S2B**).

Figure 4 – Measurement of glucose concentration in the supernatant. (**A to D**) simple *atfA-D* mutants and their complemented strains. (**E to J**) Measurement of glucose available in MM after the growth of *atfA-D* double mutants compared with the wild-type strain. Aliquots of the supernatant of three repetitions were collected at different time points (2, 4, 6, 8, 12 and 24 hours). The data were analyzed using (Prism, GraphPad) "Two-way ANOVA" followed by "Bonferroni posttests". The levels of significance were of ** $p < 0.01$ and *** $p < 0.001$ compared to the wild-type strain.

Decreased glucose consumption for the single and double mutants led us to conclude that the deletion of *atfA-D* genes impacts carbon source utilization. In the presence of acetate 1 % as single carbon source, the $\Delta atfA \Delta atfB$, $\Delta atfA \Delta atfC$, and $\Delta atfA \Delta atfD$ mutants mimic the $\Delta atfA$ 50 % growth reduction on acetate (Figures 5A and 5B, and Table 1) while $\Delta atfC$, $\Delta atfD$, and $\Delta atfC \Delta atfD$ have about 40 % increased growth on acetate compared to the wild-type strain (Figures 5A and 5B, and Table 1). In the presence of ethanol 1 % as single carbon source, the $\Delta atfC$, $\Delta atfD$, $\Delta atfB \Delta atfC$, $\Delta atfC \Delta atfD$ mutants have about 40 % growth reduction when compared to the wild-type strain (Figures 5C and 5D, and Table 1). There is an additive interaction in the $\Delta atfA \Delta atfB$ double mutant (Figures 5C and 5D, and Table 1).

Taken together, these data suggest a multi-factorial interaction between TFs AtfA-D that contributes to modulate metabolism allowing the adaptation of the fungus to stressful conditions, therefore impacting *A. fumigatus* ability to use different carbon sources.

Figure 5 – The radial growth of $\Delta atfA-D$ single and double null mutants on acetate or ethanol as single carbon sources. The wild-type and null mutants were grown for 5 days at 37 °C. The results are the means of measuring the diameter of 3 colonies for each strain $\pm$ standard deviation. The data were analyzed using (Prism, GraphPad) "Two-way ANOVA" followed by "Bonferroni posttests". The levels of significance were of ** $p < 0.01$ and *** $p < 0.001$ compared to the wild-type strain.

Accumulation of trehalose and glycogen is reduced in the double deletion mutants. We have previously shown that MAPKs Saka and MpkC physically interact with protein kinase A and these protein kinases are necessary for normal accumulation/degradation of trehalose and glycogen (de Assis et al., 2018). The $\Delta atfD$, $\Delta atfA \Delta atfD$, $\Delta atfB \Delta atfD$, and $\Delta atfC \Delta atfD$ have about 30 to 50 % reduction in trehalose accumulation when compared to the wild-type strain (**Figures 6A and 6B**, and **Table 1**). There is about a two-fold increased glycogen accumulation in $\Delta atfD$ and $\Delta atfA \Delta atfC$ when compared to the wild-type (**Figures 6C and 6D**, and **Table 1**). Interestingly, all the double mutants, except $\Delta atfA \Delta atfC$, have about 50 to 90 % glycogen reduction when compared to the wild-type (**Figures 6C and 6D**, and **Table 1**).

These data suggest that AtfA-D are important to modulate the metabolism of storage sugars, like trehalose and glycogen.

Figure 6 – There is altered trehalose and glycogen accumulation in the $\Delta atfA$ - D single and double null mutants. The strains were grown for 24 or 48 hours in MM+1% glucose. The results are the means of 3 repetitions for each strain $\pm$ standard deviation. The data were analyzed using (Prism, GraphPad) "Two-way ANOVA" followed by "Bonferroni posttests". The levels of significance were of * $p < 0.1$; ** $p < 0.01$ and *** $p < 0.001$ compared to the wild-type strain. **(A and B)** Trehalose and **(C and D)** glycogen accumulation.

The genetic interaction between the *atfA-D* genes contributes to virulence in *A. fumigatus*. The single mutants $\Delta atfA$ and $\Delta atfB$ are either attenuated or avirulent in both *Galleria mellonella* larval model and in a neutropenic murine model (Silva et al., 2017). To evaluate the virulence of double deletion mutants we used the *G. mellonella* model of infection (Silva et al., 2017). The double mutant $\Delta atfA \Delta atfB$ is avirulent and are epistatic to $\Delta atfA$ and $\Delta atfB$ (**Figure 7A** and **Table 1**) while *atfA* or *atfB* deleted in the $\Delta atfC$ or $\Delta atfD$ mimicked the avirulent phenotypes of $\Delta atfA$ and $\Delta atfB$ (**Figures 7B-E** and **Table 1**). Interestingly $\Delta atfC \Delta atfD$ showed to be avirulent while $\Delta atfC$ suppressed the avirulent phenotype of $\Delta atfA$ (**Figure 7F** and **Table 1**).

These results suggest that AtfA-D interactions affect the *A. fumigatus* virulence in *G. mellonella*.

Figure 7 – Virulence of *A. fumigatus* $\Delta atfA-D$ single and double null mutants in *Galleria mellonella*. (**A** to **F**) Cumulative survival in the model moth *Galleria mellonella*. Larvae infection was carried out via inoculation of 10^6 conidia. For inoculations, 10 larvae were infected per wild-type and null mutant strains. The levels of significance were of * $p < 0.1$; ** $p < 0.01$ and *** $p < 0.001$ compared to the wild-type strain. Phosphate buffered saline (PBS) was used as a negative control.

SakA and TFs AtfA-D interact during the adaptative response to osmotic and cell wall stress in *A. fumigatus*. Transcriptional analysis of *A. fumigatus* identified *atfA-D* mRNA accumulation as dependent on SakA and MpkC (Silva et al., 2016). *A. nidulans* AtfA physically interacts with SakA upon oxidative stress (Lara-Rojas et al., 2011). *A. fumigatus* SakA:3xHA physically interacts not only with AtfA:GFP, but also with AtfB-D:GFP in the absence and presence of osmotic (10 min exposure to 1.2 M sorbitol) and cell wall (10 min exposure to congo red 300 $\mu\text{g/ml}$) stresses (**Figures 8A and 8B and Supplementary Figure S3**).

These results suggest that SakA is directly or indirectly interacting with AtfA-D raising the hypothesis that SakA is directly involved in the activation of TFs AtfA-D in *A. fumigatus*.

Figure 8 – Co-Immunoprecipitation (Co-IP) assays between SakA and AtfA-D. **(A)** Verification of the association between AtfA:GFP and SakA:3xHA and association between AtfB:GFP and SakA:3xHA. **(B)** Verification of the association between AtfC:GFP and SakA:3xHA and association between AtfD:GFP and SakA:3xHA. Affinity purification assays from GFP-tagged AtfA-D strain in the background of 3xHA-tagged SakA were performed with GFP-Trap and anti-HA to verify interactions upon sorbitol and Congo Red (CR). Coimmunoprecipitated proteins were analyzed for the indicated antibodies. input staining with Coomassie blue.

Co-IP reveals interactions between the *A. fumigatus* AtfA-D. We constructed six different combinations of functional strains expressing AtfA-D:GFP and AtfA-D:3xHA (AtfA:GFP AtfB:3xHA, AtfA:GFP AtfC:3xHA, AtfD:GFP AtfA:3xHA, AtfB:GFP AtfC:3xHA, AtfD:GFP AtfB:3xHA, and AtfD:GFP AtfC:3xHA; **Figure 9** and **Supplementary Figure S4**). Subsequently, we evaluated through co-IP experiments the hypothesis that AtfA-D could physically interact forming heterodimers during different stressing conditions. AtfA:GFP interacted with AtfB, AtfC, and AtfD, both at before (T0) and after the addition of sorbitol or CR (**Figures 9A-C**). The TFs AtfC and AtfD also showed physical interaction in all three conditions (**Figure 9E**). In addition, AtfB interacted with AtfC in the three conditions T0, sorbitol, and CR (**Figure 9F**).

Together, these data strongly suggest that the interactions of TFs are of great importance for efficient gene transcription, allowing the fungus to quickly and robustly respond to different stressing conditions.

Figure 9 – Co-Immunoprecipitation (Co-IP) interaction experiments between AtfA-D TFs. **(A)** Association between AtfA:GFP and AtfB:3xHA proteins. **(B)** Association between AtfA:GFP and atfC:3xHA proteins. **(C)** Association between AtfD:GFP and AtfA:3xHA proteins. **(D)** Association between AtfB:GFP and AtfC:3xHA proteins. **(E)** Association between AtfD:GFP and AtfB:3xHA proteins. **(F)** Association between AtfD:GFP and AtfC:3xHA. Affinity purification assays from GFP-tagged AtfA:GFP; AtfB:GFP and AtfD:GFP strain in the background of AtfA-, B- and C:3xHA-tagged were performed with GFP-Trap and anti-HA to verify interactions upon sorbitol and Congo Red (CR). Coimmunoprecipitated proteins were analyzed for the indicated antibodies. input staining with Coomassie blue.

Discussion

The exposure of fungal cells to different stressing conditions leads to the activation of MAPK cascades which are involved in maintenance and modulation of the adaptative response, resulting in increased resistance and ensuring the survival of the fungus. Signaling through MAPK cascades results in altered gene expression that regulates many processes, including pheromone response, filamentous growth, cell wall component biosynthesis, establishment of virulence, and mediation of drug resistance (Bahn et al., 2005; Valiante et al., 2008, 2009; De Castro et al., 2014). The *A. fumigatus* HOG pathway encompasses a MAPK cascade activated by phosphorylation and is triggered by stresses such as osmotic, oxidative, and damage to the cell wall, eventually resulting in the activation of the effector kinases SakA^{Hog1p} and MpkC (Bruder- Nascimento et al., 2016). These protein kinases can then migrate to the nucleus and regulate the activity of additional protein targets, thus modulating a cellular response to the extracellular environment (Bruder-Nascimento et al., 2016). Our group previously showed that in *A. fumigatus* the expression of the TFs AtfA and AtfB during osmotic stress was dependent on SakA^{Hog1p}, while AtfC and AtfD were dependent on both MpkC and SakA^{Hog1p}. Furthermore, *atfA-D* null mutants are more sensitive to oxidative and cell wall stresses (Silva et al., 2017). In the present work, we studied possible genetic and protein interactions between AtfA-D. Our results indicate that there are several levels of genetic interactions among these TF mutants. However, *atfA* and *atfB* null mutants displayed epistasis in most of the stressing conditions here investigated. The single mutants $\Delta atfA$ and $\Delta atfB$ showed reduced virulence in *G. mellonella* while $\Delta atfC$ and $\Delta atfD$ were as virulent as the wild-type. However, genetic interactions only between $\Delta atfC$ and $\Delta atfD$ have decreased virulence in *G. mellonella* since the other double mutants are impacted by either $\Delta atfA$ or $\Delta atfB$. Some AtfA-D homologues have already been characterized in other *Aspergilli*. In *A. niger*, AtfC homologue (An12g10230) has been shown to be involved in the formation of conidia and resistance to stress (Leeuwen et al., 2013). In addition, the *A. niger atfC* showed high levels of transcription in dormant conidia and strong negative regulation during germination (Leeuwen et al., 2013). The AtfB homologue in the *A. oryzae* is involved in many cellular processes, such as stress tolerance and amino acid

metabolism (Sakamoto et al., 2008). In *A. parasiticus*, AtfB is a master regulator of three functional networks, secondary metabolism, stress response and conidiospore development (Wee et al., 2017). In *A. nidulans*, AtfB homologue is related to conidia production since the deletion of *atfB* generated a strain whose conidia numbers were slightly reduced when compared to the wild-type (Sakamoto et al., 2008). These data indicate the involvement of AtfA, AtfB and AtfC during adaptation to osmotic and cell wall stress and in the production of conidia. In *A. fumigatus*, the reduction of conidiation was not observed for strains with single deletions or for strains with double deletions.

In *S. cerevisiae*, Hog1 kinase converts the Sko1-Cyc8-Tup1 repressor complex into an activator in response to osmotic stress (Proft and Struhl, 2002). *S. cerevisiae* Sko1p is a bZIP TF of the ATF/CREB family that targets approximately 40 genes with different biological functions, including response to hyperosmotic transcriptional stress (Rep et al., 2001; Proft et al., 2005). In *Schizosaccharomyces pombe*, activation of the MAPK pathway leads to phosphorylation of the MAPK HOG Spc1/Sty1^{Hog1p}, which then accumulates in the nuclei, promoting activation and transcriptional repression of genes partially or completely dependent on *A. fumigatus* AtfA homologue Atf1p (Shiozaki K and Russell, 1995; Millar et al., 1995; Wilkinson et al., 1996; Kawasaki et al., 2002). *S. pombe* Atf1 is important for the response to various stressing conditions and has the ability to form a heterodimer with another bZip TF, Pcr1p (Lawrence et al., 2007; Salat-Canela et al., 2017), which then participates in meiotic recombination, maintenance of the heterochromatin structure and gene regulation (Salat-Canela et al., 2017). Upon oxidative stress *A. nidulans* SakA is translocated to the nucleus and also physically interacts with AtfA, whose location is constitutively nuclear (Lara- Rojas et al., 2011; Kato et al., 2013; Salat-Canela et al., 2017).

In mammalian cells, the formation of heterodimers by TFs bZip type are suggested to control a large number of regulatory transcriptional systems, increasing the diversity of responses and the regulatory potential determined by TFs (Wolberger, 1998; Rodriguez-Martinez et al., 2017). Surprisingly, we observed a complex interaction among all four AtfA-D forming heterodimers and suggesting this possibility in *A. fumigatus*. In addition, we have demonstrated that

A. fumigatus SakA physically interacts not only with AtfA but also with AtfB, -C and -D. The TF AtfA^{Atf1p} of *A. fumigatus* interacts directly with sakA^{hog1p} upon different stress conditions. In addition, we observed association among AtfB, AtfC and AtfD with SakA^{hog1p}. These data indicate that the four transcription factors are dependent on SakA, further revealing that the four TFs interact physically with the SakA^{hog1p} during transcriptional activation. In *A. fumigatus*, AtfA is also constitutively present in the nuclei, regardless of the stressful conditions to which the fungus is exposed. In contrast, only a fraction (~ 10%) of AtfB-D:GFP is located in the nuclei. However, after exposure to osmotic, oxidative or cell wall stresses, migration of AtfB, -C and -D to the nucleus occurs in approximately 80%. These results indicate that the mechanism of activation of AtfB-D is through initial translocation to the nucleus while AtfA is already present in the nucleus. All these proteins are possibly functionally activated by phosphorylation by their interaction with SakA. It remains to be investigated if SakA directly phosphorylates AtfA-D and what are the AtfA-D targets that are modulated during these stressing conditions.

We observed the single and double $\Delta atfA-D$ mutants are more susceptible to cell wall damaging agents, suggesting an altered cell wall composition. The cell wall of *A. fumigatus* is composed of α - and β -glucans, chitin, galactomannans and also the precursor of N-acetylglycosamine (NAG). Glucose is the most abundant cell wall monosaccharide, representing 50% of all fungal cell wall polysaccharides (de Assis et al., 2015). As an initial step to understand if AtfA-D are involved in the carbon source metabolism and could affect the biosynthesis of the cell wall polysaccharides, we decided to observe the growth of these mutants in different carbon sources and how they accumulate storage sugars such as trehalose and glycogen. The main carbon sources available in the host for pathogenic fungi are glucose, lactate and acetate (Ries et al., 2018). At low levels of glucose and nutrients, acetate plays an important role in maintaining energy homeostasis in mammals and can be derived from several different sources (Riaz et al., 2016), such as, if obtained exogenously from food or formed by the intestinal microbiota, later being absorbed and used in metabolism. Acetate can also be produced endogenously from the metabolism of ethanol, deacetylation of acetylated proteins or acetylcholine A (Buckley and William,

2015). Acetate is involved in virulence, for example, in the bacterium *Escherichia coli* that has a system capable of assimilating acetate as a means of survival and replication within macrophages (Setta et al., 2012; Cole and Nizet, 2016; Plovier and Cani, 2017). *A. fumigatus* is capable of using different carbon sources and this characteristic is important for its survival within the host and, therefore, for virulence. The single and double mutants *atfA-D* mutants showed a reduction in glucose transport and growth in the presence of glucose as single carbon source. Interestingly, the mutant $\Delta atfA$ showed a reduction in its ability to metabolize acetate while $\Delta atfC$ and $\Delta atfD$ have increased growth in the presence of acetate as single carbon source compared to the wild-type strain. In contrast, $\Delta atfC$ and $\Delta atfD$ have decreased growth in the presence of glycerol as single carbon source.

Trehalose is a non-glucose-reducing disaccharide synthesized by insects, plants, bacteria and fungi. As a reserve carbohydrate, trehalose is found in resting cells and spores, and may constitute up to 15% of the dry weight of these structures (Wmkler and kienle, 1991). For fungi, trehalose is an important source of energy, being used during cellular processes such as glycolysis, sporulation and germination. In addition, trehalose protects cells, preventing the aggregation of denatured proteins, promoting the elimination of free radicals, in addition to helping cells to resist environmental stress and nutrient deprivation (Singer and Lindquist, 1998). In yeast cells, trehalose is accumulated in large quantities, serving as a reserve compound and also as a protector of cell integrity against conditions of nutritional deprivation and environmental damage such as oxidative and osmotic stress and thermal shock (Martínez- Esparza et al., 2007). Recent studies have shown that trehalose biosynthesis may be involved in mediating the response to stress and also in the virulence of pathogenic fungi (Martínez- Esparza et al., 2007) serving as an energy source during the early stages of germination and contributing to the fungal infection (de Assis et al., 2015). Surprisingly, trehalose is absent in mammalian cells and it has been suggested that enzymes involved in trehalose biosynthesis can be used as targets for antifungal therapy (Al-Bader et al., 2010). Our data showed that the single $\Delta atfD$ mutant and the double mutants $\Delta atfA \Delta atfD$, $\Delta atfB \Delta atfD$ and $\Delta atfC \Delta atfD$ reduced trehalose accumulation when compared to the wild type, suggesting that *AtfD* is important for transcriptional regulation of genes involved in trehalose

biosynthesis. The conidia of the mutant strain $\Delta atfA$, have previously been shown to reduce trehalose accumulation (Hagiwara et al., 2014). However, our results revealed that $\Delta atfA$ has normal trehalose levels in mycelia, suggesting that AtfA is important only for trehalose accumulation in conidia. The glycogen levels were also quantified and single mutants $\Delta atfC$ and $\Delta atfD$ have increased and decreased glycogen levels, respectively, when compared to the wild-type strain. Interestingly, there is a synergistic effect between $\Delta atfA$ and $\Delta atfB$ since in the double mutant there is decreased glycogen accumulation.

These results strongly suggest that AtfA-D are able to modulate genes involved in the primary metabolism, most likely genes related to carbon source assimilation. It remains to be investigated if this effect on primary carbon metabolism is indirectly affecting trehalose and glycogen accumulation, and/or if the genes involved in the metabolism of these storage sugars are directly regulated by AtfA-D.

The MAPK-HOG SakA and MpkC pathway is important for maintaining intracellular glycogen and trehalose levels, which are necessary for glucose uptake and intracellular glycemic signaling (de Assis et al., 2014). These data corroborate the data obtained by our study, showing once again that SakA interacts with TFs AtfA-D during metabolic regulation. In addition, impaired glucose uptake and use for single and double AtfA-D mutants may result in altered cell wall composition, which would explain the sensitivity of the mutants to different cell wall stressors. Protein kinase A (PKA) pathway regulation is important for the use of carbon sources and the degradation of trehalose and glycogen (Freitas et al., 2010). Therefore, our studies suggest that AtfA-D could participate in this regulatory cascade, activating or repressing important targets for carbon source assimilation and sugar storage. Our results suggest that there is a cross talking between the HOG and PKA signaling pathways, in which AtfA-D are possibly activated.

AUTHOR CONTRIBUTIONS

LPS realized formal analysis, investigation, wrote original draft, review and editing. MACH Wrote, review and editing. GHG conceptualization, funding

acquisition, project administration, supervised wrote original draft, review and editing the manuscript. All authors revised the manuscript.

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4.0 Conclusões finais

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A via de sinalização de glicerol de alta osmolaridade ativada por mitógeno (MAPK HOG) desempenha uma diversidade de papéis na biologia fúngica. Uma vez que as quinases SakA e/ou MpkC e os fatores de transcrição AtfA-D estejam ausentes, os impactos na capacidade de tolerância ao estresse e na adaptação do fungo às diferentes condições ambientais são fortemente afetados. Os impactos negativos incluem a redução de crescimento, a perda da resistência a antifúngicos e a virulência atenuada. Assim, com os resultados obtidos, podemos concluir:

- (i) O RNAseq feito para os mutantes $\Delta sakA$, $\Delta mpkC$ e para o duplo $\Delta sakA \Delta mpkC$ revelou que os fatores de transcrição AtfA-D são regulados ao nível transcripcional pelas quinases após o fungo sofrer estresse osmótico.
- (ii) Os fatores de transcrição AtfA-D contribuem para a transcrição e a modulação de genes que atuam na capacidade adaptativa de *A. fumigatus* frente a tensões de parede celular, osmóticas e oxidativas.
- (iii) Os TFs também estão envolvidos no potencial patogênico do fungo. A deleção dos genes *atfA* e *AtfB* levou à perda de virulência e à virulência atenuada, respectivamente.
- (iv) Nós também elucidamos a localização celular dos quatro fatores de transcrição, através da fusão com GFP, evidenciando que: a) AtfA:GFP está constitutivamente localizado no núcleo das células de *A. fumigatus*. b) AtfB:GFP está localizado no citoplasma, sendo translocado para o núcleo após o fungo ser exposto a condições de estresse. Esse mesmo perfil de localização foi visto para AtfC:GFP e AtfD:GFP, que migram para os núcleos em condições de estresse.

- (v) Através da técnica de co-imunoprecipitação (Co-IP), nós pudemos observar que os fatores de transcrição AtfA, -B, -C e -D interagem fisicamente com a quinase SakA.
- (vi) Os quatro fatores de transcrição interagem entre si, possivelmente formando heterodímeros, ampliando a capacidade de regulação transcricional dos genes.
- (vii) Os quatro fatores de transcrição também possuem interação genética. Após a caracterização de cepas duplo-mutantes, nós observamos maior número de interações genéticas principalmente para $\Delta atfA$ $\Delta atfB$. Entretanto, outros duplo-mutantes também apresentam interações genéticas.
- (viii) Os TFs também podem estar envolvidos na regulação bioquímica e metabólica. Essa hipótese foi levantada, após observarmos a redução do crescimento em fontes de carbono alternativa, como acetato e etanol e fontes de armazenamento de carbono, tais como trealose e glicogênio.
- (ix) Nossos dados também mostram que *A. fumigatus* possui uma maquinaria bioquímica altamente articulada, exibindo interação entre as vias de MAPK HOG, CWI e PKA.

5.0 Referências bibliográficas

5.0 Referências

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Putative Membrane Receptors Contribute to Activation and Efficient Signaling of Mitogen-Activated Protein Kinase Cascades during Adaptation of *Aspergillus fumigatus* to Different Stressors and Carbon Sources

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Putative Membrane Receptors Contribute to Activation and Efficient Signaling of Mitogen-Activated Protein Kinase Cascades during Adaptation of *Aspergillus fumigatus* to Different Stressors and Carbon Sources

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ABSTRACT The high-osmolarity glycerol (HOG) response pathway is a multifunctional signal transduction pathway that specifically transmits ambient osmotic signals. *Saccharomyces cerevisiae* Hog1p has two upstream signaling branches, the sensor histidine kinase Sln1p and the receptor Sho1p. The Sho1p branch includes two other proteins, the Msb2p mucin and Opy2p. *Aspergillus fumigatus* is the leading cause of pulmonary fungal diseases. Here, we investigated the roles played by *A. fumigatus* SlnA^{Sln1p}, ShoA^{Sho1p}, MsbA^{Msb2p}, and OpyA^{Opy2p} putative homologues during the activation of the mitogen-activated protein kinase (MAPK) HOG pathway. The *shoA*, *msbA*, and *opyA* singly and doubly null mutants are important for the cell wall integrity (CWI) pathway, oxidative stress, and virulence as assessed by a *Galleria mellonella* model. Genetic interactions of ShoA, MsbA, and OpyA are also important for proper activation of the SakA^{Hog1p} and MpkA^{Slt2} cascade and the response to osmotic and cell wall stresses. Comparative label-free quantitative proteomics analysis of the singly null mutants with the wild-type strain upon caspofungin exposure indicates that the absence of ShoA, MsbA, and OpyA affects the osmotic stress response, carbohydrate metabolism, and protein degradation. The putative receptor mutants showed altered trehalose and glycogen accumulation, suggesting a role for ShoA, MsbA, and OpyA in sugar storage. Protein kinase A activity was also decreased in these mutants. We also observed genetic interactions between SlnA, ShoA, MsbA, and OpyA, suggesting that both branches are important for activation of the HOG/CWI pathways. Our results help in the understanding of the activation and modulation of the HOG and CWI pathways in this important fungal pathogen.

IMPORTANCE *Aspergillus fumigatus* is an important human-pathogenic fungal species that is responsible for a high incidence of infections in immunocompromised individuals. *A. fumigatus* high-osmolarity glycerol (HOG) and cell wall integrity pathways are important for the adaptation to different forms of environmental adversity such as osmotic and oxidative stresses, nutrient limitations, high temperatures, and other chemical and mechanical stresses that may be produced by the host immune system and antifungal drugs. Little is known about how these pathways are activated in this fungal pathogen. Here, we characterize four *A. fumigatus* putative homologues that are important for the activation of the yeast HOG pathway. *A. fumigatus* SlnA^{Sln1p}, ShoA^{Sho1p}, MsbA^{Msb2p}, and OpyA^{Opy2p} are genetically interacting and are essential for the activation of the HOG and cell wall integrity pathways. Our results contribute to the understanding of *A. fumigatus* adaptation to the host environment.

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Aspergillus fumigatus causes aspergillosis, which includes chronic pulmonary aspergillosis (CPA), allergic bronchopulmonary aspergillosis (ABPA), and invasive pulmonary aspergillosis (IPA) (1). IPA has a mortality rate of 50% to 75% when treated, affecting primarily immunocompromised individuals, patients with cancer or hematological neoplasms, or patients undergoing chemotherapy (1–3). *A. fumigatus* is able to adapt to different forms of environmental adversity such as osmotic and oxidative stresses, nutrient limitations, high temperatures, and other chemical and mechanical stresses, some of which may be produced by the host immune system and antifungal drugs (4). The survival capacity of *A. fumigatus* under different stress conditions depends on its response and adaptation mechanisms (4).

The high-osmolarity glycerol (HOG) response pathway is a multifunctional signal transduction pathway that specifically transmits ambient osmotic signals (5). The HOG pathway is activated by mitogen-activated protein kinases (MAPKs) during adaptation to environmental stress and morphology regulation (5). The MAPK cascades consist of three kinases, MAPK, MAPK kinase (MAPKK), and MAPKK kinase (MAPKKK), which are highly conserved in fungi (6–8). After the environmental stimulus, the MAPK cascade is activated by sequential phosphorylation, resulting in the activation of transcription factors and expression of target genes that assist in the adaptation to a given condition (5, 9). In *Saccharomyces cerevisiae*, Hog1p has the following two upstream signaling branches (9–11) (Fig. 1): (i) the Sho1p (synthetic high osmolarity) membrane protein, containing four N-terminal transmembrane domains (TM) and one C-terminal domain (SH3) (9, 11, 12), and (ii) Sln1p (synthetic lethal of N-end rule), a transmembrane histidine phosphotransfer kinase and osmosensor with an intracellular kinase domain forming a phosphorelay system similar to bacterial two-component regulators (9, 11). In yeast, Sho1p is responsible for transmitting signals of osmotic stress by sequentially activating the MAPKKs Pbs2p and Hog1p. In addition, the Sho1p branch includes two other TM proteins, an Msb2p (multicopy suppressor of a budding defect) mucin and an Opy2p (overproduction-induced pheromone-resistant yeast) type 1 TM ancestral protein (13, 14). Under conditions of hyperosmotic stress, these TM proteins form a complex that is composed of two GTPases (Cdc42p and Cdc24p) and three kinases (Ste11p, Ste50p, and Ste20p) and that activates the Hog1p pathway (Fig. 1) (15–17).

The Sln1 branch of the yeast HOG pathway is an example of a two-component system (11, 18, 19). The typical organization of a two-component system consists of the following components: (i) a sensor histidine kinase (SHK) that contains an input (or sensor) domain, an HK catalytic domain, and a histidine autophosphorylation site and (ii) a response regulator (RR) that contains a receiver (REC) domain and an output (or effector) domain (20). The sensor domain is modified by a stimulus, a histidine close to the HK domain is phosphorylated (or dephosphorylated), and this phosphoryl group is transferred to the REC domain of the RR (20). Yeast has three REC proteins (Sln1, Ssk1, and Skn7), one SHK (Sln1), and one HPT (Ypd1) (Fig. 1). *S. cerevisiae* Sln1 is responsible for the coordination of two distinct signaling pathways: the Sln1-Ypd1-Ssk1 phosphorelay pathway, which is important for the regulation of hyperosmolarity responses, and the Sln1-Ypd1-Skn7 pathway, which is important for the regulation of hypo-osmolarity responses (11).

In *A. fumigatus*, ShoA, the putative homologue of *S. cerevisiae* Sho1p, has been shown to be important for fungal morphology and oxidative stress; however, a thorough investigation of ShoA function has not been performed (21). The *A. fumigatus* Sln1p homologue has been previously characterized (22). The $\Delta tcsB^{slnA/sln1p}$ mutant was partially sensitive to SDS, and Western blot analysis of both the corresponding wild-type strain and the $\Delta tcsB^{slnA/sln1p}$ mutant showed that when stressed with hydrogen peroxide, phosphorylation of Hog1p still occurred in the mutant (22). The *A. fumigatus* Msb2p mucin homologue, MsbA, has been shown to be important for fungal devel-

FIG 1 The two branches of Sln1 and Sho1 which are responsible for the activation of the *S. cerevisiae* high-osmolarity glycerol (HOG) pathway. (Adapted from reference 43).

opment, biofilm formation, and cell wall integrity (CWI) (23). The function of the TM Opy2p putative homologue in *A. fumigatus* (here named OpyA) has not been elucidated, and the interactions of the ShoA branch with the MsbA and OpyA and SlnA^{Sln1p} branches during HOG MAPK pathway activation are undefined.

Four MAPKs have been identified in *A. fumigatus*: (i) MpkA, which is important for the CWI pathway and oxidative stress (22, 24, 25); (ii) MpkB, which regulates the pheromone response/filamentous growth pathway, conidiation, and dihydroxynaphthalene (DHN) melanin production (26, 27); and (iii) Saka and (iv) MpkC, which are paralogues that regulate the HOG pathway (28). MpkC and Saka interact physically and play roles in caspofungin tolerance and carbon source utilization, respectively (24, 29–33). We do not have very much information about the upstream mechanisms by which MpkC and Saka are activated and execute their signaling functions.

Here, we investigated in more detail the roles played by both the Sln1 and Sho1 *A. fumigatus* putative branches during the activation of the MAPK HOG pathway. Our results strongly indicate that these four proteins actively collaborate to promote activation of osmotic stress responses and the cell wall integrity pathway (CWI) MpkA MAPK in *A. fumigatus*.

RESULTS

The Sho1 branch: functional characterization of three *A. fumigatus* genes encoding putative receptors. *A. fumigatus* ShoA and MsbA homologues were previously identified (21, 23). We validated their organization and identified *A. fumigatus* putative homologues of *S. cerevisiae* Opy2p. (i) ShoA^{Sho1p} (*A. fumigatus* B_055960 [AFUB_055960]) has 311 amino acids and 30% identity and 48% similarity with Sho1p (E value = 4e–39). It has four transmembrane regions and a Src homology 3 (SH3) domain (Interpro IPR001452) that binds to target proteins through sequences containing proline and hydrophobic amino acids (Fig. 2A). (ii) MsbA^{Msb2p} (AFUB_098950) has 901 amino acids and 34% identity and 55% similarity with Msb2p (E value = 8e–12). It has a putative signal peptide (from amino acids 1 to 21) and a single transmembrane region at the C terminus (Fig. 2B). (iii) OpyA^{Opy2p} (AFUB_034820) has 432 amino acids

FIG 2 *A. fumigatus* ShoA, MsbA, and OpyA. (A to C) Protein organization of *A. fumigatus* ShoA (A), MsbA (B), and OpyA (C). (D to J) Growth phenotypes of the wild-type strain and the $\Delta shoA$, $\Delta msbA$, $\Delta opyA$, $\Delta shoA::shoA^+$, $\Delta msbA::msbA^+$, and $\Delta opyA::opyA^+$ mutants. The strains were

(Continued on next page)

TABLE 1 Summary of the growth inhibition of the wild-type strain and *slnA*, *shoA*, *msbA*, and *opyA* single and double mutants grown under different stressing conditions

Strains/ Phenotypes	Wild-type	$\Delta shoA$	$\Delta msbA$	$\Delta opyA$	$\Delta shoA$ $\Delta msbA$	$\Delta msbA$ $\Delta opyA$	$\Delta shoA$ $\Delta opyA$	$\Delta slnA$	$\Delta slnA$ $\Delta shoA$	$\Delta slnA$ $\Delta msbA$	$\Delta slnA$ $\Delta opyA$
CR											
CFW											
CaCl ₂											
Sorbitol											
Menadione											
Caspofungin											
CPE											
Virulence											
MM+acetate								ND	ND	ND	ND
MM+glycerol								ND	ND	ND	ND
MM+ethanol								ND	ND	ND	ND

	Growth Inhibition (%)
	0 to 20
	21 to 40
	41 to 60
	61 to 80
	81 to 100

*CR, Congo red (50 μ g/ml); CFW, calcofluor white (90 μ g/ml); CaCl₂ (500 mM); Sorbitol 1.2 M; Menadione 0.05 M; Caspofungin (2 μ g/ml); CPE, Caspofungin Paradoxical Effect (8 μ g/ml); Virulence, virulence in *Galleria mellonella*; MM+acetate 1%; MM+glycerol 1%; MM+ethanol 1%; ND, not determined.

and very low identity with Opy2p that is restricted to the Opy2 domain that acts as a membrane anchor in the HOG signaling pathway (Interpro IPR018571, from amino acids 22 to 56) and a single transmembrane region located at the N terminus (Fig. 2C).

Null mutant strains for *shoA*, *msbA*, and *opyA* were constructed and were functionally complemented by homologous integration (Fig. 2D and E; see also Fig. S1 at https://figshare.com/articles/Membrane_receptors_contribute_to_activation_and_efficient_signaling_of_Mitogen-Activated_Protein_Kinase_cascades_during_adaptation_of_AspERGILLUS_fumigatus_to_different_stressors_and_carbon_sources/12402125). They had about 15% reduced growth compared to wild-type strain in YAG (except for the $\Delta opyA$ mutant, which showed the same radial diameter as the wild-type strain) and minimal medium (MM) (Fig. 2D and E). They were also phenotypically characterized under different stress conditions such as cell wall damage and osmotic and oxidative stresses (Fig. 2F to K). All three mutants were more sensitive to Congo red (CR), calcofluor white (CFW), CaCl₂, sorbitol, and menadione than the wild-type and corresponding complementing strains (Fig. 2F to J). All three mutants were more sensitive to caspofungin, an echinocandin that noncompetitively inhibits β -1,3-glucan synthase, impairing fungal cell wall polysaccharide biosynthesis and integrity (Fig. 2K) (24, 34). Caspofungin paradoxical effect (CPE) is described as a phenomenon where high caspofungin concentrations restore the expected inhibition of *A. fumigatus* growth (35). All three mutants lost the CPE (Fig. 2K). A summary of the phenotypes is shown in Table 1. These results indicate that ShoA, MsbA, and OpyA are important for the activity of the CWI pathway and for responses to osmotic and oxidative stresses.

ShoA, MsbA, and OpyA null mutants have genetic interactions. To investigate possible genetic interactions between these putative receptors, we constructed $\Delta shoA$ $\Delta msbA$, $\Delta shoA$ $\Delta opyA$, and $\Delta msbA$ $\Delta opyA$ double mutants (Fig. 3A and B). The double mutants showed about 15% reduced growth compared to the wild-type strain in YAG and MM (Fig. 3A and B). All of the double mutants showed levels of susceptibility to CR 50 μ g/ml similar to those seen with the corresponding single mutants (Fig. 3C), suggesting that *shoA*, *msbA*, and *opyA* are functioning in the same pathway to repair and/or process cell wall damage caused by CR (Tables 1 and 2). In contrast, all the

FIG 2 Legend (Continued)

grown for 4 days at 37°C (D) on MM or YAG (E), MM plus Congo red (CR) (F), MM plus calcofluor white (CFW) (G), MM plus CaCl₂ (H), MM plus sorbitol (I), MM plus menadione (J), and MM plus caspofungin (K), and their radial growth was quantified. The results shown represent the means of measurements of the diameter of 3 colonies for each strain $\pm$ standard deviation. The data were analyzed (Prism, GraphPad) using two-way ANOVA followed by Bonferroni posttests. The levels of significance compared to the wild-type results are indicated as follows: *, $P < 0.1$; **, $P < 0.01$; ***, $P < 0.001$.

FIG 3 *A. fumigatus* double null mutants indicate genetic interactions among $\Delta shoA$, $\Delta msbA$, and $\Delta opyA$. Growth phenotypes of the wild-type strain and the $\Delta shoA$, $\Delta msbA$, $\Delta opyA$, $\Delta msbA \Delta opyA$, $\Delta shoA \Delta msbA$, and $\Delta shoA \Delta opyA$ mutants were (Continued on next page)

TABLE 2 Summary of the genetic interactions between *slnA*, *shoA*, and *msbA* single mutants grown under different stressing conditions

Strains/ Phenotypes ^a	$\Delta shoA \Delta msbA$	$\Delta msbA \Delta opyA$	$\Delta shoA \Delta opyA$	$\Delta slnA \Delta shoA$	$\Delta slnA \Delta msbA$	$\Delta slnA \Delta opyA$
CR						
CFW						
CaCl ₂						
Sorbitol						
Menadione						
Caspofungin						
CPE						
Virulence						
MM+acetate				ND	ND	ND
MM+glycerol				ND	ND	ND
MM+ethanol				ND	ND	ND

	epistatic interaction
	additive interaction
	suppression interaction
	no interaction

^aCR, Congo red (50 μ g/ml); CFW, calcofluor white (90 μ g/ml); CaCl₂ (500 mM); Sorbitol 1.2 M; Menadione 0.05 M; Caspofungin (2 μ g/ml); CPE, Caspofungin Paradoxical Effect (8 μ g/ml); Virulence, virulence in *Galleria mellonella*; MM+acetate 1%; MM+glycerol 1%; MM+ethanol 1%; ND, not determined.

double mutants showed increased susceptibility to CFW 90 μ g/ml in comparison to the corresponding single mutants, suggesting an additive interaction (Fig. 3D; see also Tables 1 and 2). The double mutants were also as sensitive to 500 mM CaCl₂ as the corresponding single mutants, suggesting again that *shoA*, *msbA*, and *opyA* function in the same pathway for calcium signaling (Fig. 3E). The double mutants, except the $\Delta msbA \Delta opyA$ mutant, were as sensitive to osmotic stress caused by 1.2 M sorbitol as the corresponding single mutants (Fig. 3F; see also Tables 1 and 2), suggesting once more that *shoA* and *msbA* and *shoA* and *opyA* function in the same pathway for osmotic stress signaling. However, the $\Delta msbA \Delta opyA$ mutant was more resistant to osmotic stress (Fig. 3F; see also Tables 1 and 2), suggesting that *msbA* and *opyA* do not genetically interact. The $\Delta shoA \Delta msbA$ mutant was more sensitive to oxidative stress caused by 0.05 mM menadione than the corresponding single mutants, suggesting an additive interaction (Fig. 3G; see also Tables 1 and 2) while the $\Delta msbA \Delta opyA$ and $\Delta shoA \Delta opyA$ mutants were less sensitive than the corresponding single mutants (Fig. 3G; see also Tables 1 and 2), suggesting suppression interactions. All the double mutants were as sensitive to lower caspofungin and CPE concentrations as the single mutants (Fig. 3H; see also Tables 1 and 2), suggesting that they function in the same pathway for caspofungin signaling.

Considering the paucity of genetic transformation markers for *A. fumigatus*, we did not complement the double mutants. However, we tested few of the phenotypes described above for two independent candidates of the double mutants (see Fig. S2). They showed exactly the same phenotypes, strongly indicating that there were no additional mutations that were introduced into these double mutants during the

FIG 3 Legend (Continued)

determined. (A to H) The strains were grown for 4 days at 37°C (A) on MM or YAG (B) or on MM plus Congo red (CR) (C), MM plus calcofluor white (CFW) (D), MM plus CaCl₂ (E), MM plus sorbitol (F), and MM plus menadione (G), and (H) MM plus caspofungin, and their radial growth was quantified. The results shown represent the means of measurements of the diameter of 3 colonies for each strain $\pm$ standard deviation. The data were analyzed (Prism, GraphPad) using two-way ANOVA followed by Bonferroni posttests. The levels of significance compared to the wild-type results are indicated as follows: *, $P < 0.1$; **, $P < 0.01$; ***, $P < 0.001$. (I) PCA of the results shown in panels A to E. (J and K) Detection of β -1,3-glucan (Dectin-1; J) and chitin (CFW; K) exposed on the cell surface was performed. WT, wild type. Experiments were performed in triplicate, and the results are displayed as mean values with standard errors (two-way ANOVA followed by Tukey's $P < 0.05$).

transformation process and that could be responsible for the observed phenotypes (see Fig. S2).

The differences between the mutant and wild-type strains were analyzed through a principal-component analysis (PCA) (Fig. 3I). This analysis was performed with the set of all data obtained through measurement of the radial growth of each strain under different stress conditions. In the spatial distribution of the graph, we can see that the single $\Delta shoA$, $\Delta msbA$, and $\Delta opyA$ mutants are grouped, indicating that the single mutants showed similar phenotypes after growth under different stress conditions (CR, CFW, menadione, and sorbitol). These data show that ShoA, MsbA, and OpyA have similar and/or redundant functions.

Looking at the result of the PCA for the double mutants, we can see that the $\Delta msbA \Delta opyA$ and $\Delta shoA \Delta opyA$ double mutants are similar and also grouped apart from the wild-type and the single mutants (Fig. 3H). This suggests that ShoA can fulfill some of the functions performed by MsbA in the $\Delta msbA \Delta opyA$ double mutant. Similarly, MsbA can fulfill some of the functions of ShoA in the $\Delta shoA \Delta opyA$ double mutant. As a consequence, when we subjected the double mutants to stress conditions, we observed that the $\Delta shoA \Delta opyA$ and $\Delta msbA \Delta opyA$ double mutants showed the same behavior.

Finally, we observed that the $\Delta shoA \Delta msbA$ double mutant was different from the wild-type strain, the single mutants, and the other two double mutants (the $\Delta shoA \Delta opyA$ and $\Delta msbA \Delta opyA$ mutants). This suggests that ShoA and MsbA have unique functions during signaling caused by stressful conditions. A summary of the phenotypes of and the genetic interactions between the single mutants is shown in Tables 1 and 2. The data set shows that these putative TM proteins can have redundant as well as unique functions during the activation of the signaling pathways in *A. fumigatus*.

Next, we investigated the cell wall organization using the exposure of different polysaccharides on the cell surface. All the single and double mutants showed a germling length comparable to that seen with the wild-type strains after 16 h of growth at 37°C without shaking. Dectin-1 binding results revealed increased exposure of β -glucans only in the $\Delta shoA \Delta opyA$ and $\Delta shoA \Delta msbA$ cell walls compared with the wild-type strain and all the other mutants (Fig. 3I). However, CFW staining showed that the $\Delta shoA$, $\Delta opyA$, and $\Delta msbA$ mutants exhibited an increase in chitin exposure in comparison to the wild-type and complemented strains (Fig. 3J). The double mutants once more showed a synergistic interaction because their level of chitin exposure was much higher than that of the corresponding single mutants (Fig. 3J).

Taken together, these results strongly indicate a genetic interaction among ShoA, MsbA, and OpyA in response to several stressing conditions that affect the cell wall structure and organization and responses to oxidative and osmotic stresses.

ShoA, MsbA, and OpyA null mutants influence SakA and MpkA phosphorylation. Subsequently, we investigated the impact of the deletion of the three putative receptors on the phosphorylation of MAP kinases SakA and MpkA in the presence of higher osmotic concentrations (1.2 M sorbitol) or different caspofungin concentrations (Fig. 4; for an additional repetition of the Western blot assays, see Fig. S3). These Western blot assays were semiquantitative experiments, and the results were somewhat variable. We focus on the trends that were common to the two independent experiments, though the precise fold changes might have differed between the experiments. The wild-type strain showed increased (about 4-fold) SakA phosphorylation when exposed for 10 min to 1.2 M sorbitol (Fig. 4A). In contrast, the $\Delta shoA$, $\Delta msbA$, and $\Delta opyA$ single mutants showed higher levels of SakA phosphorylation than the wild-type strain, with 11-fold, 5.4-fold, and 5-fold phosphorylation increases at 10 min, respectively (Fig. 4A). The $\Delta shoA \Delta msbA$ and $\Delta shoA \Delta opyA$ double mutants had a synergistic interaction, since both mutants showed much lower SakA phosphorylation than the corresponding single mutants whereas the $\Delta opyA \Delta msbA$ double mutant showed about 3-fold SakA phosphorylation at 10 min (Fig. 4B).

The wild-type strain exhibited increased (about 2-fold) MpkA phosphorylation when exposed to 1.2 M sorbitol for 60 min (Fig. 4A). All three single mutants show higher

FIG 4 *A. fumigatus* ShoA, MsbA, and OpyA are important for SakA and MpkA phosphorylation. Western blotting assays of SakA and MpkA phosphorylation in the wild-type strain and single mutants (A and C) or in the wild-type strain and double mutants (B and D) in response to 1.2 M sorbitol for different periods of time (A and B) and in response to caspofungin 0.25, 2.0, or 8.0 μ g/ml for 1 h (C and D) were performed. Anti-P-p38 SakA and anti-44/42 MpkA antibodies were used to detect the phosphorylation of SakA and MpkA, respectively, while anti- γ -tubulin was used to detect γ -tubulin. Signal intensities were quantified using ImageJ software, and ratios of (P)-SakA to γ -tubulin or (P)-MpkA to γ -tubulin were calculated.

induction of MpkA phosphorylation levels than the wild-type strain at 60 min (Fig. 4A). The double mutants $\Delta shoA \Delta msbA$ and $\Delta shoA \Delta opyA$ showed lower MpkA phosphorylation levels than the corresponding single mutants, while the $\Delta opyA \Delta msbA$ double mutant showed about 13-fold- and 2-fold-increased MpkA phosphorylation at 30 and 60 min exposure to sorbitol, respectively, compared to the control without sorbitol (Fig. 4B).

When exposed to 2 μ g/ml of caspofungin, the wild-type strain showed 2-fold and 2.8-fold more phosphorylation of SakA and MpkA, respectively (Fig. 4C). The $\Delta shoA$, $\Delta msbA$, and $\Delta opyA$ mutants showed no induction of either SakA or MpkA at 2 μ g/ml of caspofungin, but the $\Delta shoA$ mutant showed about 1.6-fold induction of MpkA phosphorylation at 8 μ g/ml of caspofungin (Fig. 4C). The $\Delta shoA \Delta msbA$ double mutant showed no increase in SakA and MpkA phosphorylation, while the $\Delta shoA \Delta opyA$ mutant showed about 2-fold SakA phosphorylation at 2 and 8 μ g/ml of caspofungin but no MpkA phosphorylation at any caspofungin concentration (Fig. 4D). The $\Delta opyA \Delta msbA$ double mutant showed no SakA phosphorylation in the presence of caspofungin but about 3.7-fold MpkA phosphorylation at 8 μ g/ml of caspofungin (Fig. 4D).

Taken together, these results strongly indicate that ShoA, MsbA, and OpyA can affect the levels of MpkA and SakA phosphorylation when *A. fumigatus* is exposed to osmotic and cell wall stresses.

After several attempts, we were unable to construct a $\Delta shoA \Delta msbA \Delta opyA$ triple mutant. We therefore attempted to construct a conditional *xylp::shoA* $\Delta msbA \Delta opyA$ mutant strain by replacing the *shoA* endogenous promoter with the *xylp* promoter from *Penicillium chrysogenum*, which was induced by xylose and was repressed by glucose (36) in the $\Delta msbA \Delta opyA$ strain. However, when this approach failed, we successfully replaced the *shoA* endogenous promoter with the *xylp* promoter in the wild-type strain (see Fig. S1). The *xylp::shoA* strain showed 100-fold and 600-fold increases in the levels of *shoA* transcripts when exposed for 30 and 120 min to xylose 1%, respectively, compared to the wild-type strain (Fig. 5A). *shoA* overexpression caused an almost complete reduction of growth rate in the *xylp::shoA* strain compared to the wild-type strain grown on MM plus 1% xylose (Fig. 5B). Overexpression of *shoA* also resulted in increased SakA phosphorylation (2.6-fold to 16-fold) and MpkA phosphorylation (about

FIG 5 *A. fumigatus shoA* overexpression inhibits growth. (A) The wild-type and *xylp::shoA* strains were grown in MM plus 1% glucose for 16 h at 37°C and transferred to MM plus 1% xylose for 30 to 120 min at 37°C. The results shown represent the means of measurements of the diameter of 3 colonies for each strain $\pm$ standard deviation. Gene expression was normalized using *tuba* (Afu1g10910). Standard deviations present averages of results from three independent biological repetitions (each performed with 2 technical repetitions). (B) The wild-type, *xylp::shoA*, and $\Delta shoA$ strains were grown either on MM plus 1% glucose or on MM plus 1% xylose for 5 days at 37°C. (C) The wild-type, *xylp::shoA*, and $\Delta shoA$ strains were grown in MM plus 1% glucose for 16 h at 37°C and transferred to MM plus 1% xylose for 30 to 120 min at 37°C. Western blotting assays of SakA and MpkA phosphorylation were performed. Anti-P-p38 SakA and anti-44/42 MpkA antibodies were used to detect the phosphorylation of SakA and MpkA, respectively.

2-fold) in comparison to the control (strain *xylp::shoA* grown in 1% glucose) (Fig. 5C). The levels of SakA and MpkA phosphorylation in the wild-type strain grown in xylose showed about a 2.5-fold increase and no increase, respectively, compared to the wild-type strain grown in glucose 1% (Fig. 5C). The levels of SakA and MpkA phos-

FIG 6 *A. fumigatus* *shoA*, *msbA*, and *opyA* mutants are important for virulence in *Galleria mellonella*. Cumulative survival rates of wild-type and *shoA* (A) *msbA* (B), and *opyA* (C) single mutant strains and wild-type and $\Delta shoA \Delta msbA$ (D) $\Delta shoA \Delta opyA$ (E), and $\Delta msbA \Delta opyA$ (F) double mutant strains in the model moth *Galleria mellonella* are shown. Infection of larvae was carried out via inoculation of 10^6 conidia. For inoculations, 10 larvae were infected per wild-type or null mutant strain. The levels of significance compared to the wild-type results are indicated as follows: *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$. Phosphate buffered saline (PBS) was used as a negative control.

phorylation showed no increase in the $\Delta shoA$ mutant grown in xylose 1% compared to glucose 1% (Fig. 5C).

Taken together, these results strongly indicate that ShoA, MsbA, and OpyA genetic interactions are important for proper activation of the SakA and MpkA cascade and the response to osmotic and cell wall stresses.

ShoA, MsbA, and OpyA are important for *A. fumigatus* virulence in *Galleria mellonella*. *G. mellonella* larvae were used to evaluate the importance of ShoA, MsbA, and OpyA in regulating *A. fumigatus* pathogenicity (Fig. 6). In the *G. mellonella* model, infection by the wild-type strain resulted in 100% mortality 8 days postinfection (Fig. 6). However, the $\Delta shoA$ and $\Delta msbA$ mutants showed only 60% mortality rates 10 days postinfection, which was statistically significantly different from the results seen with the wild-type strain according to Mantel-Cox and Gehan-Breslow-Wilcoxon tests ($P < 0.001$; Fig. 6A and B). The $\Delta opyA$ mutant caused 20% mortality 10 days postinfection, which was not statistically significantly different from the results seen with the phosphate-buffered saline (PBS) control according to the Mantel-Cox and Gehan-Breslow-Wilcoxon tests ($P < 0.001$; Fig. 6C). Interestingly, infection by all three double mutants resulted in 10% to 30% mortality rates 10 days postinfection, which was not statistically significantly different from the phosphate buffer saline (PBS) control results according to the Mantel-Cox and Gehan-Breslow-Wilcoxon tests ($P < 0.001$; Fig. 6D to F), suggesting that *shoA*, *msbA*, and *opyA* function in the same pathway for the establishment of virulence in *A. fumigatus* (Tables 1 and 2).

Taken together, these results clearly demonstrate that ShoA, MsbA, and OpyA play an important role in *A. fumigatus* virulence.

Proteomic analysis reveals that ShoA, MsbA, and OpyA contribute to the metabolic modulation of *A. fumigatus*. We used label-free quantitative proteomics (spectral counts) to investigate proteins that are differentially abundant in the $\Delta shoA$, $\Delta msbA$, and $\Delta opyA$ mutants upon exposure to caspofungin stress (Fig. 7; see also Table S1). We decided to expose the wild-type and the single mutants to caspofungin (8 $\mu\text{g}/\text{h}$ for 1 h) because this concentration and this exposure time are important for CPE, and all the three single mutants lost the CPE (37) (Fig. 2K). Upon exposure to caspofungin (8 $\mu\text{g}/\text{h}$ for 1 h), we observed increased abundances of 50, 109, 206, and 148 proteins and decreased abundances of 125, 130, 108, and 165 proteins in the wild-type, $\Delta shoA$, $\Delta msbA$, and $\Delta opyA$ strains, respectively (Fig. 7A). Venn diagrams were generated to correlate the protein abundance profiles for each strain treated with caspofungin (Fig. 7B). Although each strain had a unique set of proteins (135 for the wild-type strain, 94 for the $\Delta shoA$ mutant, 142 for the $\Delta msbA$ mutant, and 137 for the $\Delta opyA$ mutant), there was extensive overlap among the three mutant strains (Fig. 7B), suggesting independent and common roles for ShoA, MsbA, and OpyA in distinct pathways involved in the caspofungin response. Proteins of significant differential abundances in the $\Delta shoA$, $\Delta msbA$, and $\Delta opyA$ mutants were classified in terms of biological function. Upon caspofungin stress, in the wild-type strain there was an increase in the abundance of proteins involved in but not limited to (i) C-2 compound and organic acid metabolism and (ii) proteasomal degradation. There was also a decrease in the abundance of proteins involved in transcription and translation (Fig. 7C). In the mutant strains, there was an increase in the abundance of proteins involved in but not limited to (i) heat shock response, (ii) unfolded protein response, (iii) mitochondrial function, and (iv) protein folding and stabilization (Fig. 7D to F). In these strains, there was a reduction in the abundance of proteins belonging to categories such as (i) proteasomal degradation; (ii) sugar, glucoside, and polyol; (iii) stress response; (iv) glycolysis and gluconeogenesis; (v) pentose phosphate pathway; and (vi) C-compound and metabolism (Fig. 7D to F). Therefore, this proteomic analysis implies that upon caspofungin exposure, the absence of ShoA, MsbA, and OpyA affects the osmotic stress response, carbohydrate metabolism, and protein degradation.

ShoA, MsbA, and OpyA are important for the utilization of several carbon sources. Proteomic data from the $\Delta shoA$, $\Delta msbA$, and $\Delta opyA$ mutants revealed that many proteins involved in the catabolism of glucose and other sugars and amino acids, as well as in glucogenesis, were decreased in abundance (see Table S1). The single and double mutants showed about 10% to 15% growth reduction compared to the wild-type strain on 1% glucose (Fig. 8A), as well as reduced growth on 1% acetate, 1% glycerol, and 1% ethanol (Fig. 8B to D). For each carbon source (acetate, glycerol, or ethanol), the single and double mutants showed about the same level of growth reduction, suggesting that they function in the same pathway for assimilation of these carbon sources (Tables 1 and 2). There was also a reduction in the dry weight of the single and double mutants after 48 h in liquid MM plus 1% glucose (Fig. 9A). Glucose transport was not affected in the mutant strains (see Fig. S4), suggesting that there were no defects in glucose assimilation in these mutants. However, there was increased trehalose accumulation in the single and double mutants at 24 h or 48 h or at both time points (Fig. 9B). We also observed decreased glycogen accumulation in the $\Delta shoA$ mutant after 24 h of growth and increased glycogen accumulation in the $\Delta msbA$ mutant at 48 h of growth compared to the wild-type strain (Fig. 9C). Glycogen accumulation was decreased and increased in all three double mutants at 24 and 48 h of growth, respectively (Fig. 9C). These results suggest that glucose metabolism and sugar storage are affected in the putative receptor mutants.

We recently observed that the MAPKs SakA and MpkC are involved in the *A. fumigatus* cell wall integrity pathway and interact with protein kinase A (PKA) to control carbohydrate mobilization for cell wall remodeling (38). Protein kinase A activity in the single and double mutants was comparable to that seen with the wild-type strain at

24 h of growth but was significantly reduced after 48 h of growth in glucose 1% (Fig. 10). Taken together, these results strongly indicate that the ShoA, MsbA, and OpyA putative receptors can affect carbon source utilization and protein kinase A activity.

The Sln1 branch: genetic interactions between the two branches. An *A. fumigatus* Sln1p homologue was previously identified (22). We validated its organization as follows: SlnA^{Sln1P} (AFUA_2G00660, also called TcsB, www.aspgd.org) has 1,096 amino acids and 43% identity and 63% similarity with Sln1p (E value = 2e−40); it has two transmembrane regions, a HisKA domain (Interpro IPR003661) and a His kinase A phosphoacceptor domain (from amino acid 553 to amino acid 618); HATPase_c (Interpro IPR003594), a histidine kinase-like ATPse (from amino acid 696 to amino acid 863); and a REC domain (Interpro001789), a CheY-homologous receiver domain (from amino acid 960 to amino acid 1077) (Fig. 11A).

The Δ slnA mutant has about 15% reduced growth compared to the wild-type strain in YAG and MM (Fig. 11B and C). The Δ slnA mutant is more sensitive to sorbitol (1.2 M), CaCl₂ (500 mM), and caspofungin (0.5 to 2.0 μ g/ml) and more resistant to CFW (30 μ g/ml) than the wild-type and complemented strains but not to CFW, CaCl₂, and menadione (Fig. 11D to I); however, the Δ slnA mutant retained the CPE (Fig. 11I). In the *G. mellonella* model, infection by the wild-type and Δ slnA::slnA⁺ strains resulted in 100% mortality 8 and 9 days postinfection, respectively (see Fig. S5), while the Δ slnA mutant showed a 70% mortality rate 10 days postinfection, which was not statistically significantly different from the rates seen with the wild-type and complemented strains according to Mantel-Cox and Gehan-Breslow-Wilcoxon tests ($P < 0.001$; see Fig. S5). The double mutants Δ slnA Δ shoA, Δ slnA Δ msbA, and Δ slnA Δ opyA were as virulent as the single mutants Δ shoA, Δ msbA, and Δ opyA, suggesting there are no genetic interactions between slnA and shoA, msbA, and opyA for the establishment of virulence in *A. fumigatus* (Tables 1 and 2).

To investigate possible genetic interactions between the SlnA and ShoA branches, we constructed the double mutants Δ slnA Δ shoA, Δ slnA Δ msbA, and Δ slnA Δ opyA. They had about 10% to 15% reduced growth compared to the wild-type strain in YAG and MM (Fig. 11B and C). Only the double mutant Δ slnA Δ msbA was susceptible to 50 μ g/ml CR, suggesting there were no genetic interactions with the single mutants in the presence of CR (Fig. 11J; see also Tables 1 and 2). In contrast, all of the double mutants were more sensitive to 90 μ g/ml CFW than the corresponding single mutants, suggesting an additive interaction between slnA and shoA, msbA, and opyA (Fig. 11K; see also Tables 1 and 2). All the double mutants were as sensitive to 500 mM CaCl₂ and 1.2 M sorbitol as the corresponding single mutants, suggesting that slnA, shoA, msbA, and opyA function in the same pathway for calcium and osmotic stress signaling (Fig. 11L and M; see also Tables 1 and 2). The Δ slnA Δ shoA mutant was less sensitive to 0.05 mM menadione than the corresponding single mutants, suggesting suppression interactions between slnA and shoA, while the slnA and msbA mutants and slnA and opyA mutants were more sensitive to menadione than the corresponding single mutants, suggesting an additive interaction (Fig. 11N; see also Tables 1 and 2). There were no genetic interactions between slnA and shoA, msbA, and opyA, since the Δ slnA double mutants were more resistant to lower and CPE caspofungin concentrations than the corresponding single mutants (Fig. 11O; see also Tables 1 and 2).

We also investigated the impact of slnA deletion on the phosphorylation levels of the MAP kinases SakA and MpkA in the presence of higher osmotic concentrations (1.2 M sorbitol) of CR instead of caspofungin as an alternative agent for mediating cell wall damage (Fig. 12; for an additional repetition of the Western blot assays, see Fig. S3).

FIG 7 Legend (Continued)

mutants when exposed to caspofungin 2 μ g/ml for 1 h. (B) Venn diagrams comparing the abundances of proteins in the wild-type and each mutant strain. (C to F) A summary of the FunCat terms overrepresented with respect to increased or decreased abundance (adjusted P value of <0.05) in the wild-type strain exposed to caspofungin and compared to the control (C), in the Δ shoA mutant versus the wild-type strain posttransfer to caspofungin (D), in the Δ msbA mutant versus the wild-type strain posttransfer to caspofungin (E), and in the Δ opyA mutant versus the wild-type strain posttransfer to caspofungin (F). For the full list, refer to Table S1.

FIG 8 The $\Delta shoA$, $\Delta msbA$, and $\Delta opyA$ single and double null mutants showed reduced growth on glucose 1% (A), acetate 1% (B), glycerol 1% (C), or ethanol 1% (D) as a single carbon source. The wild-type and null mutant strains were grown for 5 days at 37°C. The results shown represent the means of measurements of the diameter of 3 colonies for each strain $\pm$ standard deviation. The data were analyzed (Prism, GraphPad) using two-way ANOVA followed by Bonferroni posttests. The levels of significance compared to the wild-type results are indicated as follows: *, $P < 0.1$; **, $P < 0.01$; ***, $P < 0.001$.

FIG 9 Altered trehalose and glycogen accumulation in the $\Delta shoA$, $\Delta msbA$, $\Delta opyA$, and double null mutants. The strains were grown for 24 or 48 h in MM plus 1% glucose. The results shown represent the means of measurements of the diameter of 3 colonies for each strain $\pm$ standard deviation. (A) Dry weight. (B) Trehalose accumulation. (C) glycogen accumulation. The data were analyzed (Prism, GraphPad) using two-way ANOVA followed by Bonferroni posttests. The levels of significance compared to the wild-type results are indicated as follows: *, $P < 0.1$; **, $P < 0.01$; ***, $P < 0.001$.

FIG 10 Protein kinase A activity was reduced in the $\Delta shoA$, $\Delta msbA$, $\Delta opyA$, and double null mutants. The strains were grown for 24 or 48 h in MM plus 1% glucose. The results shown represent the means of measurements of three independent repetitions $\pm$ standard deviation. The data were analyzed (Prism, GraphPad) using two-way ANOVA followed by Bonferroni posttests. The levels of significance compared to the wild-type results are indicated as follows: *, $P < 0.1$; **, $P < 0.01$; ***, $P < 0.001$.

The wild-type strain showed increased SakA phosphorylation (about 4-fold) when exposed for 10 min to 1.2 M sorbitol (Fig. 12A). The $\Delta slnA$ mutant showed a level (about 4-fold at 10 min) of SakA phosphorylation comparable to that seen with the wild-type strain (Fig. 12A). In contrast, there was reduced SakA phosphorylation in the $\Delta slnA \Delta msbA$ and $\Delta slnA \Delta opyA$ strains whereas there was about a 5.4-fold induction of SakA phosphorylation at 10 min of exposure in the $\Delta slnA \Delta shoA$ strain (Fig. 12A). There was about 2-fold- and 2.3-fold-increased MpkA phosphorylation in the wild-type and $\Delta slnA$ strains in the presence of 1.2 M sorbitol, while the $\Delta slnA \Delta msbA$ strain showed increases of at least 7.1-fold and 24.8-fold in MpkA phosphorylation (Fig. 12A). The $\Delta slnA \Delta shoA$ strain exposed to 1.2 M sorbitol for 30 and 60 min showed about 4.1-fold and 13.2-fold phosphorylation (Fig. 12A).

When exposed to CR (300 μ g/ml), the wild-type strain showed about 1.7-fold and 1.4-fold more phosphorylation of SakA and MpkA, respectively, at 10 min (Fig. 12B). The increase in SakA phosphorylation in the $\Delta slnA$ mutant was comparable to that seen with the wild-type strain (about 1.9-fold) in the presence of CR, but no such increase in MpkA phosphorylation was seen (Fig. 12B). There were increases in SakA phosphorylation in the $\Delta slnA \Delta msbA$ mutant of about 2.4-, 2.1-fold, and 3.4-fold in the presence of CR at 10, 30, and 60 min, respectively, and a late increase in SakA phosphorylation (about 1.9-fold at 60 min) in the $\Delta slnA \Delta shoA$ mutant (Fig. 12B). There was no MpkA phosphorylation induction seen either in the $\Delta slnA$ single mutant or in the double mutants (Fig. 12B).

Taken together, these results strongly indicate that the SlnA and ShoA branches have genetic interactions that influence growth under several stressing conditions and are important for the proper activation of the SakA and MpkA cascade and the response to osmotic and cell wall stresses.

DISCUSSION

The ability of pathogenic fungi to survive hostile environments inside and outside the host depends on their ability to respond quickly and robustly to stress (39). MAPK signaling pathways are highly conserved, promoting fungal adaptation to stress by activating the phosphorylation cascades of kinases, which in turn accumulate in the nucleus, triggering adequate cellular responses to different stimuli (40). The *S. cerevisiae*

FIG 11 *A. fumigatus* SlnA and ShoA branches are genetically interacting. (A) Protein organization of *A. fumigatus* SlnA. (B to O) Growth phenotypes of the wild-type strain and the Δ slnA, Δ shoA, Δ msbA, Δ opyA, Δ slnA Δ shoA, Δ slnA Δ msbA, and Δ slnA Δ opyA mutants were determined. The strains were grown for 4 days at 37°C (B) on MM and YAG (C), MM plus Congo red (CR) (D and J), MM plus calcofluor white (CFW) (E and K), MM plus CaCl₂ (F and L), MM plus sorbitol (G and M), MM plus menadione (H and N), and MM plus caspofungin (I and O), and their radial growth was quantified. The results shown represent the means of measurements of the diameter of 3 colonies for each strain $\pm$ standard deviation. The data were analyzed (Prism, GraphPad) using two-way ANOVA followed by Bonferroni posttests. The levels of significance compared to the wild-type results are indicated as follows: *, $P < 0.1$; **, $P < 0.01$; ***, $P < 0.001$.

HOG pathway has already been widely characterized (41, 42). Sho1p and Sln1p are two independent upstream branches that activate the HOG pathway (11). Hkr1, Msb2, and Opy2 interact with Sho1, activating the Ste20-Ste11-Pbs2-Hog1 kinase cascade (9, 11, 43). The Sln1p branch is important for the regulation of hyperosmolarity and hypo-osmolarity responses (11).

Further studies are needed to understand how *A. fumigatus* activates and controls its HOG pathway. The main objective of this study was to characterize the *A. fumigatus* putative homologues of the different components of the yeast HOG pathway activation branches Sln1p and Sho1p. Accordingly, we characterized four putative genetic determinants involved in these two branches: SlnA^{Sln1p}, ShoA^{Sho1p}, MsbA^{Msb2p}, and OpyA^{Opy2p}. Apparently, there is no *A. fumigatus* Hkr1p homologue. We have shown that all four genes are not essential and that the Δ slnA mutant is not sensitive to any kind of investigated stress (except osmotic stress) or involved in virulence, while ShoA, MsbA, and OpyA are important for the cell wall integrity pathway and for oxidative

FIG 11 (Continued)

stress and virulence. The construction and preliminary phenotypic characterization of $\Delta shoA$, $\Delta msbA$, and $\Delta slnA$ mutants were previously described (21–23). Most of the phenotypes described for the $\Delta shoA$ and $\Delta msbA$ mutants (21, 23) were also observed here. However, the growth of the $\Delta slnA$ mutant described here was more sensitive to osmotic stress than that of the $\Delta slnA$ strain previously described (22). Since the two studies used mutants derived from the same strain, differences could have been due to the growth conditions or medium composition.

The $shoA$, $msbA$, and $opyA$ null mutants showed genetic interactions under stressing conditions, indicating that they interact for the activation of stress response mechanisms (Table 2). Although ShoA, MsbA, and OpyA have similar and/or redundant functions, they have unique functions during the activation of the signaling pathways in *A. fumigatus*. Interestingly, double mutants of the three genes in the Sho1p branch with $\Delta slnA$ show genetic interactions to different extents, strongly suggesting the two pathways interact in response to stress conditions. The modulation of MAPK HOG SakA and CWI MpkA phosphorylation levels is altered in these mutants, indicating that these two branches are important for activation of not only HOG but also CWI MAPKs. We cannot discard the possibility that these receptors also affect the MAPK phosphatases, altering the global levels of SakA and MpkA dephosphorylation. We made several unsuccessful attempts to construct triple null mutants with the three putative TM

FIG 12 The *A. fumigatus* SlnA branch is important for SakA and MpkA phosphorylation. Western blotting assays of SakA and MpkA phosphorylation in response to 1.2 M sorbitol (A) and Congo red 300 μ g/ml (B) were performed for different periods of time. Anti-P-p38 SakA and anti-44/42 MpkA antibodies were used to detect the phosphorylation of SakA and MpkA, respectively, while anti- γ -tubulin was used to detect γ -tubulin. Signal intensities were quantified using ImageJ software, and ratios of (P)-SakA to γ -tubulin or (P)-MpkA to γ -tubulin were calculated.

receptors. We were also unable to construct a triple mutant with a conditional *xylp::shoA* gene in the $\Delta msbA \Delta opyA$ background under both repressing and inducing conditions. A single *xylp::shoA* mutant strain was not able to grow in xylose and induced high SakA and MpkA phosphorylation levels. These results provide direct evidence that the ShoA and SlnA branches are able to modulate activities of MAPKs.

In yeast, genetic analyses showed that the *S. cerevisiae* Sho1-Opy2 interaction enhances the activation of HOG Pbs2 MAPKKK by Fus3 MAPKK Ste11 but not the activation of Hog1 by Pbs2 (43). The interaction among these putative TM receptors has also been observed in many different human- and plant-pathogenic fungi. In the pathogenic fungus *Candida albicans*, *SLN1* deletion impaired hyphal formation and attenuated virulence (44). *C. albicans* *SHO1* mutants are also sensitive to oxidative stress and to CR and CFW and are defective in morphogenesis (45). *C. albicans* Msb2p is a global regulator of temperature stress (46), while *OPY2* mutants display susceptibility to CR, but it did not play a role in the adaptation to high osmolarity or oxidative stress and virulence in the *Galleria mellonella* model (47). Sho1 and Msb2 homologues play complementary but distinct roles in stress responses, differentiation, and pathogenicity of the human pathogen *Cryptococcus neoformans* (48). In the dimorphic plant-pathogenic fungus *Ustilago maydis*, Sho1 and Msb2-like proteins play a key role during

appressorium differentiation and are involved in plant surface sensing (49). In the plant pathogen *Verticillium dahliae*, Sho1 acts as an osmosensor and is required for plant penetration and melanin biosynthesis (21). A *Fusarium oxysporum* $\Delta msb2$ mutant was shown to be essential for pathogenicity in tomato and hypersensitive to cell wall targeting compounds (50). *F. oxysporum* $\Delta sho1$ mutants were found to be hypersensitive to CFW, and a $\Delta msb2 \Delta sho1$ double mutant was even more sensitive. *F. oxysporum* Sho1 and Msb2 have converging functions activating the Fmk1 MAPK CWI cascade, promoting invasive growth and plant infection, and regulating the CWI pathway (51). These results emphasize the complex roles played by these putative TM receptors and Sln1p homologues in pathogenic fungi.

It is well documented that the response of *A. fumigatus* to caspofungin involves the combined action of HOG SakA and CWI MpkA MAPK (30, 37, 52). We investigated the protein spectral counts for the wild-type strain and the three putative TM receptor null mutants under unstressed conditions and when exposed to caspofungin. Our results revealed a completely novel aspect of the function of the putative TM receptors: their influence on growth on different carbon sources. The putative TM receptor null mutants showed reduced growth on several carbon sources compared to the wild-type strain. Although they do not have defects in glucose transport, they exhibit aberrant sugar storage metabolism, a strong indicator of problems in carbon assimilation metabolism. Cell wall-related sugars are derived from carbohydrate storage compounds during stress of the cell wall, and this process is dependent on cAMP-dependent protein kinase A (PKA) and on MAPKs SakA and MpkC (38). These protein kinases are important for normal accumulation/degradation of storage sugars through SakA physical interactions with the catalytic and regulatory subunits of protein kinase A PkaC1 and PkaR (38). The putative TM receptor mutants have reduced protein kinase A activity, and the effects on SakA phosphorylation could be the main reason for the reduced growth on several carbon sources.

In summary, we have provided novel information about *A. fumigatus* SlnA^{Sln1p} and ShoA^{Sho1p} branches and their function in the activation of HOG MAPK pathway. Interestingly, these putative receptors also affect the CWI MpkA pathway. Phosphorylation of CWI MpkA MAPK in response to osmotic and cell wall stress was found to be dependent on HOG MpkC and SakA MAPKs (28, 33). These results indicated that there is a collaboration between the CWI and HOG pathways during cell wall biosynthesis (28, 33). Our work opens new possibilities for understanding the pathways for the activation and maintenance of osmolarity and the cell wall integrity in *A. fumigatus*. Those pathways are promising targets for drug development and could help to improve the efficacy of antifungal treatment and aspergillosis treatment.

MATERIALS AND METHODS

Strains and culture medium. The strains were cultured at 37°C in complete yeast glucose (YG, 2% [wt/vol] glucose, 0.5% [wt/vol] yeast extract, trace elements) or minimal medium (MM; 1% [wt/vol] original salts with high nitrate content, trace elements, pH 6.5). For YAG and MM solid media, 1.7% (wt/vol) or 2% (wt/vol) agar were added to YG and MM. Where necessary, uracil and uracil (1.2 g/liter) were added. The strains used in this work are described in Table S2 at https://figshare.com/articles/Membrane_receptors_contribute_to_activation_and_efficient_signaling_of_Mitogen-Activated_Protein_Kinase_cascades_during_adaptation_of_AspERGILLUS_fumigatus_to_different_stressors_and_carbon_sources/12402125.

Construction of *A. fumigatus* null mutants. *A. fumigatus* strain CEA17 was used to generate the following deletion strains used in this study: the $\Delta msbA$, $\Delta opyA$, and $\Delta shoA$ single mutants, with sequences corresponding to *msbA* (Afu4g04070), *opyA* (Afu3g14410), and *shoA* (Afu2g00660) according to the AspGD database (<http://www.aspgd.org/>). The deletion cassettes were constructed by *in vivo* recombination in *S. cerevisiae* as described previously (53). We used approximately 1.0 kb of the 5' untranslated region (5'-UTR) and the 3'-UTR flanking the target open reading frame (ORF) regions was selected for primer design (see Table S3). Primers 5'Fw and 3'Rv contained a short sequence homologous to plasmid pRS426 and the *pyrG* gene. Both the 5'-UTR and 3'-UTR fragments were amplified by PCR from *A. fumigatus* genomic DNA (gDNA). The *pyrG* gene placed inside the cassette as a prototrophic marker was amplified from plasmid pCDA21. The deletion cassette was generated by transformation into *S. cerevisiae* SC94721 using the lithium acetate method (54), plus the fragments, together with plasmid pRS426 digested at two sites with enzymes BamHI and EcoRI (New England Biolabs Ltd., United Kingdom). The DNA of the transformants was extracted by a previously described method (55). The deletion cassettes were amplified by PCR from these plasmids using TaKaRa *Ex Taq* DNA polymerase

(Clontech TaKaRa Bio) and were used for the transformation of *A. fumigatus*. The same protocol was used for the construction of the double mutants. However, the marker gene used for the deletion of the gene was amplified from plasmid pPTRI. The colonies of null mutants were selected by purification of the colonies in 1 µg/ml pyrithiamine hydrobromide (Sigma). The results of Southern blot analysis demonstrated that the transformation cassette was homologously integrated (see Fig. S1). Single gene deletion strains were complemented by cotransformation of approximately 1.0 kb of the 5'-UTR and 3'-UTR plus ORF together with plasmid pPTRI. The complementants were selected by purification of colonies in MM with 1 µg/ml pyrithiamine hydrobromide. Gene complementation was checked by PCR (see Fig. S1). All the primers used in this work are described in Table S3.

Construction of *xylP::shoA* strain. The strain of *A. fumigatus* (CEA17) with *pyrG* auxotrophic mutant marker was used to generate strain *xylP::shoA*, whose *shoA* gene was fused to the *xylP* promoter, activated by xylose. The cassettes were constructed by *in vivo* recombination in *S. cerevisiae* (53), and we used about 1.0 kb of the 5'-UTR flanking region of the region and 3'-UTR of the target ORF regions were selected for the design of the primer. The *xylP* promoter was amplified from the plasmid pYES-pXyl-hph-devR. The *pyrG* gene placed inside the cassette as a prototrophic marker was amplified from plasmid pOB430. The fragments necessary for the construction of the cassette were amplified by PCR using the primers listed in Table S3. The construction of the cassette was performed *in vivo* using *S. cerevisiae* SC9721 and the lithium acetate method (54), plus the fragments, together with plasmid pRS426 digested in two sites with the enzymes BamHI and EcoRI (New England Biolabs Ltd., United Kingdom). The DNA of the transformants was extracted using a previously described method (55). The *xylP::shoA* construct cassette was amplified by PCR from these plasmids using TaKaRa Ex Taq DNA polymerase (Clontech TaKaRa Bio) and used for the transformation of *A. fumigatus*. The colonies of the transformants were selected by purification in MM (1% glucose). Candidates were checked by PCR using 5'-UTR primers external to the cassette used for transformation (see Fig. S1).

Characterization of mutants. Sensitivity and resistance to several stressing agents were evaluated by measurement of radial growth, comparing the mutant and wild-type strains. A 5-µl volume of a suspension of 2×10^7 conidia was inoculated into MM with or without a corresponding stressing agent, and the plates were incubated for 4 days at 37°C. After this period, the radial diameters were measured and the values were used for statistical analysis. The data were analyzed (Prism, GraphPad) using two-way analysis of variance (ANOVA) followed by Bonferroni posttests. The levels of significance compared to the wild-type results are indicated as follows: *, $P < 0.1$; **, $P < 0.01$; ***, $P < 0.001$.

Immunoblot analysis. The strains were inoculated (1×10^7 conidia) in 250-ml Erlenmeyer flasks with 50 ml of YAG medium and grown in a rotatory shaker (200 rpm) at 37°C for 16 h before being exposed to different stressing agents, such as Congo red, hydrogen peroxide, sorbitol, and calcium chloride. The proteins were extracted as described previously (56) and quantified according to a previously described method (57). A 60-µg volume of total protein per sample was run on a 12% (wt/vol) SDS-PAGE gel before being transferred to a polyvinylidene difluoride (PVDF) membrane (GE Healthcare). The phosphorylated forms of MpkA and SakA were detected using anti-phospho-p44/42 MAPK and anti-phospho-p38 MAPK (Cell Signaling Technology) antibodies, respectively. Primary antibodies were detected using an anti-rabbit IgG antibody, horseradish peroxidase (HRP) antibody (catalog no. 7074; Cell Signaling Technologies). The detection (chemiluminescence) was performed using a Western ECL Prime (GE Healthcare) blot detection kit according to the manufacturer's instructions. The phosphorylated signal of MpkA and SakA was normalized to the monoclonal antibody IgG γ -tubulin (C-11, sc-17787) ratio. The images generated were submitted for densitometric analysis using ImageJ software (<http://rsbweb.nih.gov/ij/index.html>).

Enzymatic assays. Analyses of intracellular trehalose levels (Megazyme) and PKA activity (Promega) were carried out according to the manufacturer's instructions. Intracellular glycogen levels and extracellular glucose concentrations were quantified as described previously (58).

Cell wall staining. Cell wall surface polysaccharide staining was performed as described previously (28, 59, 60). Briefly, strains were grown from 2.5×10^3 spores in 200 µl of MM for 16 h at 37°C before the culture medium was removed and germings were UV irradiated. The hyphal forms were subsequently washed with PBS 1×, and 200 µl of a blocking solution (2% [wt/vol] goat serum, 1% [wt/vol] bovine serum albumin [BSA], 0.1% [vol/vol] Triton X-100, 0.05% [vol/vol] Tween 20, 0.05% [vol/vol] sodium azide, and 0.01 M PBS) was added. Samples were incubated for 30 min at room temperature. For dextrin staining, 0.2 µg/ml of Fc-h-dextrin-hFc was added to the UV-irradiated germings and incubated for 1 h at room temperature, followed by the addition of 1:1,000 DyLight 594-conjugated, goat anti-human IgG1 for 1 h at room temperature. The hyphal forms were washed with PBS, and fluorescence was read at 587 nm excitation and 615 nm emission. For chitin staining, 200 µl of a PBS solution with 10 µg/ml of calcofluor white (CFW) was added to the UV-irradiated hyphal forms, incubated for 5 min at room temperature, and washed three times with PBS before fluorescence was read at 380 nm excitation and 450 nm emission. All experiments were performed using 12 repetitions, and fluorescence was read in a microtiter plate reader (SpectraMax i3; Molecular Devices).

Virulence analysis in *Galleria mellonella* models. The *Galleria mellonella* larvae were obtained by breeding adult larvae (61) weighing 275 to 330 mg, kept starving in petri dishes at 37°C in the dark for 24 h prior to infection. All selected larvae were in the final (sixth) stage of larval development. Fresh conidia of each strain of *A. fumigatus* were counted using a hemocytometer, and the initial concentration of the suspensions of conidia used for the infections was 2×10^8 conidia/ml. A total of 5 µl (1×10^6 conidia/larva) of each suspension was inoculated per larva. The control group was composed of larvae inoculated with 5 µl of PBS to observe death by physical trauma. The inoculation through the last left

proleg was performed using a Hamilton syringe (model 7000.5 KH). After infection, the larvae were kept at 37°C in petri dishes in the dark and scored daily. Larvae were considered dead due to lack of movement in response to touch. The viability of the inoculum administered was determined by plating a serial dilution of the conidia on YAG medium and incubating the plates at 37°C for 72 h. All the experiments were performed in parallel and repeated twice.

Purification and identification of proteins by LC-MS/MS. To precipitate crude protein extracts, they were prepared from *ΔshoA*, *ΔopyA*, and *ΔmsbA* mutant and wild-type cultures that were grown for 24 h and exposed to caspofungin 8 μg/ml (60 min). Crude mycelium protein extracts were obtained by extraction of ground mycelium by the use of B250 buffer (250 mM NaCl, 100 mM Tris-HCl [pH 7.5], 10% glycerol, 1 mM EDTA, and 0.1% NP-40) supplemented with 1.5 ml/liter 1 M dithiothreitol (DTT), 2 tablets/100 ml complete EDTA-free mini-protease inhibitor cocktail (Roche), 3 ml/liter 0.5 M benzamidine, 10 ml/liter phosphatase inhibitors 100× (10 M NaF, 5 M sodium vanadate, 8 M β-glycerol phosphate), and 10 ml/liter of 100 mM phenylmethylsulfonyl fluoride (PMSF). Total protein lysates were centrifuged at 13,000 rpm at 4°C for 10 min, and the supernatant was collected in a new Eppendorf tube. Samples were quantified by the Bradford method (62) and subjected to trypsinization by the use of trypsin (Promega; catalog no. 9PIV511) according to the manufacturer's instructions. The peptides obtained after trypsinization were bound to a resin (C18 ZipTip pipette tips) according to the manufacturer's instructions (Merck). Digested peptides were separated using reverse-phase liquid chromatography with an Ultimate 3000 NanoLC system (Dionex Corporation, Sunnyvale, CA, USA) followed by mass identification using a Q Exactive mass spectrometer (Thermo Fisher Scientific). Samples were loaded by the use of an autosampler onto a C₁₈ trap column, which was switched on-line with an analytical BioBasic C₁₈ PicoFrit column (C₁₈ PepMap; Dionex) (75-μm inside diameter [i.d.] by 500 mm, 2 μm particle size, 100 Å pore size). Full scans in 300 to 1,700 *m/z* were recorded with a resolution of 140,000 (*m/z* 200) with a blocking mass set to 445.12003. Protein identification and label-free quantification (LFQ) normalization were performed using MaxQuant v1.5.2.8 quantitative proteomic software (www.maxquant.org/) in conjunction with Perseus v1.5.6.0 statistical analysis software (www.maxquant.org/). ANOVA-based multisample *t* tests were performed using a *P* value cutoff point of <0.05, allowing the identification of differentially abundant, statistically significant proteins.

RNA extraction and gene expression analysis. Wild-type and *xylP::shoA* strains were inoculated (1×10^7 conidia) in 50 ml of MM for 24 h at 37°C. Mycelia were transferred to MM plus 1% xylose for 30 to 240 min. Mycelia were ground to a fine powder in liquid N₂, and total RNA was extracted with TRIzol reagent (Thermo Scientific) according to the manufacturer's protocol. DNA was digested with Turbo DNase I (Ambion Thermo Scientific) according to manufacturer's instructions. A 2-μg volume of total RNA per sample was subjected to reverse transcription with a High Capacity cDNA reverse transcription kit (Thermo Scientific) using a blend of oligo(dT) and random primers, according to the manufacturer's instructions. Quantitative real-time PCRs (qRT-PCRs) were run in a StepOne Plus real-time RT-PCR system (Thermo Scientific) using Power Sybr green PCR master mix (Thermo Scientific). Three independent biological replicates were used, and the mRNA quantity relative fold change values were calculated using standard curves (63). All values were normalized to the expression of the *A. fumigatus tubA* gene. The primers are described in Table S3.

Data availability. The proteomic data set can be accessed in Table S1 at https://figshare.com/articles/Membrane_receptors_contribute_to_activation_and_efficient_signaling_of_Mitogen-Activated_Protein_Kinase_cascades_during_adaptation_of_AspERGILLUS_fumigatus_to_different_stressors_and_carbon_sources/12402125.

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The *Aspergillus fumigatus* Phosphoproteome Reveals Roles of High-Osmolarity Glycerol Mitogen-Activated Protein Kinases in Promoting Cell Wall Damage and Caspofungin Tolerance

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ABSTRACT The filamentous fungus *Aspergillus fumigatus* can cause a distinct set of clinical disorders in humans. Invasive aspergillosis (IA) is the most common life-threatening fungal disease of immunocompromised humans. The mitogen-activated protein kinase (MAPK) signaling pathways are essential to the adaptation to the human host. Fungal cell survival is highly dependent on the organization, composition, and function of the cell wall. Here, an evaluation of the global *A. fumigatus* phosphoproteome under cell wall stress caused by the cell wall-damaging agent Congo red (CR) revealed 485 proteins potentially involved in the cell wall damage response. Comparative phosphoproteome analyses with the $\Delta sakA$, $\Delta mpkC$, and $\Delta sakA \Delta mpkC$ mutant strains from the osmotic stress MAPK cascades identify their additional roles during the cell wall stress response. Our phosphoproteomics allowed the identification of novel kinases and transcription factors (TFs) involved in osmotic stress and in the cell wall integrity (CWI) pathway. Our global phosphoproteome network analysis showed an enrichment for protein kinases, RNA recognition motif domains, and the MAPK signaling pathway. In contrast to the wild-type strain, there is an overall decrease of differentially phosphorylated kinases and phosphatases in $\Delta sakA$, $\Delta mpkC$, and $\Delta sakA \Delta mpkC$ mutants. We constructed phosphomutants for the phosphorylation sites of several proteins differentially phosphorylated in the wild-type and mutant strains. For all the phosphomutants, there is an increase in the sensitivity to cell wall-damaging agents and a reduction in the MpkA phosphorylation upon CR stress, suggesting these phosphosites could be important for the MpkA modulation and CWI pathway regulation.

IMPORTANCE *Aspergillus fumigatus* is an opportunistic human pathogen causing allergic reactions or systemic infections, such as invasive pulmonary aspergillosis in immunocompromised patients. The mitogen-activated protein kinase (MAPK) signal-

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ing pathways are essential for fungal adaptation to the human host. Fungal cell survival, fungicide tolerance, and virulence are highly dependent on the organization, composition, and function of the cell wall. Upon cell wall stress, MAPKs phosphorylate multiple target proteins involved in the remodeling of the cell wall. Here, we investigate the global phosphoproteome of the $\Delta sakA$ and $\Delta mpkC$ *A. fumigatus* and high-osmolarity glycerol (HOG) pathway MAPK mutants upon cell wall damage. This showed the involvement of the HOG pathway and identified novel protein kinases and transcription factors, which were confirmed by fungal genetics to be involved in promoting tolerance of cell wall damage. Our results provide understanding of how fungal signal transduction networks modulate the cell wall. This may also lead to the discovery of new fungicide drug targets to impact fungal cell wall function, fungicide tolerance, and virulence.

KEYWORDS *Aspergillus fumigatus*, cell wall integrity pathway, MpkC, SakA, caspofungin, osmotic stress

Aspergillus fumigatus is a filamentous fungus that can cause disease in humans (1). Depending on a patient's immunological status, *A. fumigatus* can cause a distinct set of clinical disorders that extend from severe allergies to lethal disseminated infections (1). Invasive aspergillosis (IA) is the most common life-threatening fungal disease in immunocompromised humans, and mortality rates can reach 90% (2–6). Disseminated fungal infections are treated with antifungal drugs, including polyenes, azoles, and echinocandins (7). However, infections by antifungal drug-resistant pathogens are on the rise, presenting severe treatment constraints (8). Several factors are important for *A. fumigatus* infection and survival in the human host, including hypoxia resistance, iron assimilation, gliotoxin production (depending on the immune status of the host), presence of dihydroxynaphthalene (DHN) melanin, and thermophily (9–19). How these genetic traits are integrated in response to environmental cues is poorly understood; thus, a better understanding of stress response signaling networks involved in virulence is essential for the development of improved IA treatments.

The highly conserved mitogen-activated protein kinase (MAPK) signaling pathways are essential for adaptation to environmental changes (20, 21). The MAPK cascades are important for relaying, integrating, and amplifying intracellular signals and are crucial signaling components involved in many cellular processes (20, 21). In filamentous fungi, the conserved cell wall integrity (CWI), pheromone response/filamentous growth, high-osmolarity glycerol (HOG), and MAPK pathways have been shown to influence numerous virulence traits, including invasive growth, biofilm formation, mycotoxin production, and antifungal tolerance (22–24).

A. fumigatus has four MAPKs: (i) MpkA is the central regulator of CWI pathway and also plays a role in oxidative stress tolerance (25, 26), and several other cellular processes (27); (ii) MpkB regulates the pheromone response/filamentous growth pathway, which is important for conidiation and dihydroxynaphthalene (DHN) melanin production (28); and (iii) SakA and MpkC are paralogues that constitute the main regulators of the HOG pathway (29). The MpkC and SakA protein sequences are very similar, and they play a role in caspofungin tolerance and carbon source utilization, respectively (25, 30, 31). In *Aspergillus nidulans* and *A. fumigatus*, MpkC and SakA physically interact, but also show distinct and shared functions during stress responses and development (32–34). However, little is known about the mechanisms by which MpkC and SakA execute their signaling functions.

Fungal cell survival is highly dependent on the organization, composition, and function of the cell wall. The *A. fumigatus* cell wall is essential for cell shape, virulence, and prevention of cell lysis due to strong osmotic pressures. In addition, it plays a critical role in adhesion, host recognition, and evasion of the mammalian immune system (35–37). Although the cell wall is rigid, it is also a dynamic structure and is constantly being remodeled during fungal growth and development (38–40). Echinocandins, which inhibit the biosynthesis of the cell wall component β -1,3-glucan, are

among the most recent antifungal drugs to be developed (3) and highlight that cellular integrity is a good target for further development of antifungals. Cell wall biosynthesis and the CWI pathway therefore provide excellent targets for further antifungal drug development.

The *A. fumigatus* CWI pathway has already been characterized, and it is mainly composed of the PkcA-MpkA-Rlm1 module (36, 41–45). Recently, we demonstrated that the HOG MAPs SakA and MpkC are independently required to mount an adequate response to cell wall damage (29). The $\Delta sakA$ and the double $\Delta sakA \Delta mpkC$ mutants were more sensitive to osmotic and oxidative stresses and to cell wall-damaging agents. Both MpkC:GFP (green fluorescent protein) and SakA:GFP translocated to the nucleus upon osmotic and cell wall stress, with SakA:GFP showing a quicker response. The phosphorylation of MpkA from the CWI pathway in response to osmotic and cell wall stress was dependent on MpkC and SakA, suggesting that both the CWI and HOG pathways collaborate upon exposure to several types of stresses and during cell wall biosynthesis (29, 34).

“Omics” high-throughput techniques such as genomics, transcriptomics, metabolomics, and proteomics have revolutionized biological research. Phosphoproteomics is a branch of proteomics important for the characterization of proteins that are modified posttranslationally by the addition of a phosphate group. Phosphorylation is a reversible protein modification that can regulate cell signaling networks through the regulation of protein function, subcellular localization, complex formation, and protein degradation (46). It is estimated that 30% to 65% of all proteins may be phosphorylated (46, 47). Phosphoproteomics, in particular, has been utilized to elucidate novel signaling mechanisms (48). Phosphoproteomics provides information about which protein or pathway might be activated, since generally there is a correlation between a change in protein phosphorylation and protein activity. Phosphoproteomics has been used for understanding mitotic exit and cell wall integrity pathways in *Saccharomyces cerevisiae* (49, 50); hyphal morphogenesis in *Candida albicans* (51); asexual development and multiple stress responses in *Beauveria bassiana* (52); cellular processes involved in morphology, secretion, protein kinase A regulation, and catabolite repression in *A. nidulans* (53, 54); and the aflatoxin production in *Aspergillus flavus* (55). Here, we evaluate the global *A. fumigatus* phosphoproteome exposed to cell wall-damaging agents, identifying 485 proteins putatively involved in the cell wall stress response. Comparative phosphoproteome analyses of the wild-type (WT) and $\Delta sakA$, $\Delta mpkC$, and $\Delta sakA \Delta mpkC$ mutant strains showed the involvement of the HOG pathway and identified novel protein kinases and transcription factors, which were confirmed by fungal genetics to be involved in promoting CWI signaling and tolerance of cell wall damage.

RESULTS

***A. fumigatus* phosphoproteome upon cell wall damage.** A phosphoproteomics study was used as a first step to understand how protein phosphorylation is involved in the response to the cell wall damage. To achieve this, mycelia were collected from liquid minimal medium before (T0) and after adding 200 mM Congo red (CR) for 30 min (CR30). From the wild-type, $\Delta sakA$, $\Delta mpkC$, and $\Delta sakA \Delta mpkC$ strains, at T0 and CR30, total protein was extracted, trypsin digested, subjected to TiO₂ phosphopeptide enrichment, and analyzed by liquid chromatography-tandem mass spectrometry (LC-MS/MS), as illustrated in Fig. 1A.

Principal-component analyses of the wild-type phosphoproteome showed the cluster of untreated samples separated from those exposed to CR (Fig. 1B). A total of 4,499 phosphopeptides were identified with “localization probabilities” higher than 0.75 (75% [see Table S1A in the supplemental material]). A total of 756 phosphopeptides were found to be significantly different between the untreated T0 and the CR-treated CR30 sample (Student *t* test, $P < 0.05$), corresponding to 485 proteins (Fig. 1C). Among these phosphopeptides, three were identified only in T0 samples and 380 were exclusive to CR-treated samples while 373 were observed in both treatments (Fig. 1E and Table S1B).

FIG 1 Phosphorylation profile of proteins from *A. fumigatus* wild type upon exposure to Congo red (CR 10 min). (A) Workflow of experimental design for phosphoproteome results. (B) Principal-component analysis plot for the *A. fumigatus* wild type without CR treatment (T = 0, T0WT; six replicates) and with 10-min CR treatment (CRWT; four replicates). (C) Number of phosphopeptides and unique proteins modified by phosphorylation during CR treatment. (D) Volcano plot of total phosphopeptides identified, showing a major increase of phosphorylation after CR treatment. FC, fold change. (E) Venn diagram of total 756 phosphopeptides indicating the profile of total phosphorylation (380) or total dephosphorylation (3) in wild-type strains. (F) Number of phosphosites in serine (S), threonine (T), or tyrosine (Y) for total phosphopeptides. (G) Number of phosphorylation sites for each peptide.

The distribution of phosphosites according to amino acid residues followed a pattern typical of eukaryotic organisms, with 99% occurring on serine and threonine and <1% on tyrosine residues (Fig. 1F and Table S1B). Approximately half of the phosphoproteins contained a single phosphorylation site, and phosphoproteins containing one to three phosphosites accounted for the vast majority of phosphoproteins detected (Fig. 1G and Table S1B).

FIG 2 Functional categorization and identification of kinase substrates. (A) Phosphopeptide functional categorization of approximately 75% of proteins with known function. (B) Profile of distribution of kinase families that are able to phosphorylate the 756 phosphopeptides identified by LC-MS/MS as predicted by Group-based Prediction System 3.0 (GPS; with medium-stringency cutoff). (C) Consensus sequence of the site of phosphorylation of p38/Hog1 peptide substrates predicted by Group-based Prediction System 3.0 (GPS; with medium-stringency cutoff). (D and E) Phosphorylation distribution of all kinases (D) and transcription factors (TFs) (E) modulated by phosphorylation during Congo red response. Gray blocks indicate lack of the phosphopeptide, while green (P) indicates the presence of a phosphopeptide.

FunCat (<https://sbi.hki-jena.de/fungifun/fungifun.php>) analyses for the 485 proteins showed an enrichment for proteins involved in the regulation of C-compound, translation initiation, osmosensing and response, Ca²⁺-mediated signal transduction, MAPK kinase (MAPKKK) cascades, stress responses, encoded protein kinases, actin cytoskeleton, and budding cell polarity and filament formation (Fig. 2A). This implies that the phosphorylation response to cell wall damage involves stress responses, translation, protein kinases, and different MAPK and calcium-mediated signaling mechanisms.

TABLE 1 Genes encoding protein kinases and transcription factors that showed phosphorylation when the wild-type strain is exposed to Congo red for 30 min

Protein	Putative function
Protein kinases	
HriA (AFUB_032300)	Eukaryotic translation initiation factor 2 alpha kinase activity and role in G ₁ cell cycle arrest in response to nitrogen starvation, negative regulation of cytoplasmic translational initiation in response to stress
SrrB (AFUB_030660)	Response regulator of a two-component phosphorelay system
Kin1 (AFUB_010510)	Serine/threonine protein kinase involved in regulation of exocytosis
SnfA (AFUB_018770)	Ortholog(s) has AMP-activated protein kinase activity, ADP ribosylation factor guanyl-nucleotide exchange factor activity
CmkA (AFUB_029320)	Calcium/calmodulin-dependent protein kinase
Prr1 (AFUB_029820)	Serine/threonine protein kinase; inhibits pheromone-induced signaling downstream of MAPK, possibly at the level of the Ste12p transcription factor
PkhA (AFUB_036500)	Phosphoinositide-dependent protein kinase
CmkC (AFUB_053520)	Calmodulin-dependent protein kinase activity
KfsA (AFUB_014350)	Checkpoint kinase; required for normal septation in conidiophores, not hyphae; present in hyphae and conidiophores at septa and cortex, not at spindle pole bodies
Oca2 (AFUB_059390)	Role in negative regulation of transcription by RNA polymerase II, regulation of nitrogen utilization, response to salt stress
FpkA (AFUB_059090)	Ribosomal S6 kinase (RSK)
Transcription factors	
AFUB_004210	Unknown function
AFUB_009270	Unknown function
AsgA (AFUB_019790)	Zinc cluster protein proposed to be a transcriptional regulator involved in the stress response
AreB (AFUB_029020)	GATA zinc finger transcription factor, leucine zipper motif, negative regulator of nitrogen catabolism
FkhA (AFUB_037220)	Forkhead family transcription factor; rate-limiting activator of replication origins; evolutionarily conserved regulator of lifespan; major role in expression of G ₂ /M-phase genes

Possible kinase groups targeting each of the 756 phosphorylation sites were predicted by Group-based Prediction System 3.0 (GPS; with medium-stringency cutoff) (Fig. 2B). Based on kinase-substrate relationships, seven serine/threonine kinase families accounted for most of the identified phosphorylation sites (Fig. 2B). The most frequent serine/threonine kinase families (more than 50%) were AGC, TK, and atypical, followed by the CMGC family (15%), which includes MAPKs, such as the HOG (p38) MAPK (Fig. 2B). For example, a frequently observed phosphoserine/threonine kinase was p38 (CMGC/MAPK/p38/Hog) (Fig. 2C). In fact, the analysis of the substrate sequences phosphorylated by p38 MAPKs indicates a high conservation of a serine (S) and a proline (P) in positions +8 and +9 (>90%) among the putative targets of p38 MAPK (Fig. 2C).

Protein kinases and transcription factors involved in the cell wall damage. We have visually inspected and identified the phosphopeptides that corresponded to the 32 protein kinases and 27 transcription factors (TFs) identified with increased phosphorylation upon CR exposure (Fig. 2D and E; Table S1C and D). Eleven candidate protein kinase and 5 TF-encoding genes were randomly selected (Table 1), and it was verified whether their corresponding single-gene deletion mutants were more sensitive to cell wall-damaging agents CR, calcofluor white (CFW), and caspofungin and to high-sorbitol-induced osmotic stress. No significant growth differences were observed among these protein kinase null mutants compared to the wild-type strain (Fig. S1A to D). However, the TF null mutants lacking the gene AFUB_004210, AFUB_009970, AFUB_019790, or AFUB_037220 were more sensitive to CR (Fig. 3A), while mutants lacking AFUB_004210, AFUB_009970, and AFUB_037220 were more sensitive to calcofluor white (CFW), than the wild-type strain (Fig. 3B). All the TF null mutants showed some extent of increased sensitivity to caspofungin (Fig. 3C). The TF null mutants for AFUB_009970 and AFUB_019790 were also more sensitive to osmotic stress (Fig. 3D).

None of the protein kinase null mutants lost the caspofungin paradoxical effect (CPE; the escape of *A. fumigatus* from caspofungin inhibition at concentrations above the MIC) (3). In contrast, three TF null mutants corresponding to AFUB_009970, AFUB_019790, and AFUB_037220 showed a significantly decreased CPE. Therefore, this

FIG 3 Phosphoproteomics allows the identification of novel transcription factors involved in the CWI pathway. *A. fumigatus* conidia (1×10^5) were inoculated on solid minimal medium (MM) with different concentrations of Congo red (CR), calcofluor white (CFW), caspofungin (CASPO), and sorbitol and grown for 5 days at 37°C. All plates were grown in triplicate, and the average $\pm$ SD was plotted. A Student *t* test using Prism GraphPad (version 6) was applied to confirm the statistical significance difference between treatment and control (*P* value < 0.05, ***; < 0.01, **; < 0.001, *).

phosphoproteomic study has facilitated the identification of novel protein kinases and TFs involved in osmotic, cell wall, and caspofungin stress responses.

***A. fumigatus* HOG MAPKs are important for the phosphoproteomic response to cell wall damage.** We previously demonstrated that the MpkC and SakA kinases of the

HOG pathway play a role in the response to osmotic, oxidative, and cell wall stresses, in addition to influencing CWI MpkA signaling (29). To assess the involvement of MpkC and SakA in the modulation of phosphorylation cascades activated by cell wall damage, the phosphoproteome of the $\Delta sakA$, $\Delta mpkC$, and double $\Delta sakA \Delta mpkC$ mutants was examined in the absence (T0) or presence of CR for 30 min (CR30).

When the CR30-treated $\Delta sakA$ mutant was compared to CR30-treated WT, a total of 4,835 phosphopeptides were identified (location probability >75% [Table S2A]); 409 phosphopeptides corresponding to 268 proteins show significant differences between mutant and WT (Student *t* test with $P < 0.05$) (Fig. 4A). A prevalence of phosphorylation events was observed (67 phosphopeptides with total phosphorylation exclusive for the mutant strain and 284 with increased phosphorylation), with only 33 phosphopeptides with total dephosphorylation (exclusive to WT) and 25 with decrease of phosphorylation in the mutant strain (Fig. 4A and Table S2B). For the same comparison without CR treatment (T0), a total of 3,741 phosphopeptides were selected (location probability >75%) with only 27 phosphopeptides significantly different between $\Delta sakA$ and WT strains ($P < 0.05$) (Table S3A and B).

For the comparison between $\Delta mpkC$ and WT strains after 30-min CR incubation, 4,638 phosphopeptides were identified (location probability >75% [Table S4A]); 157 phosphopeptides corresponding to 118 proteins were observed with significant differences between mutant and WT (Student *t* test with $P < 0.05$) (Fig. 4A and Table S4B). The phosphorylation profile of the $\Delta mpkC$ strain was the opposite of $\Delta sakA$ strain, with a major reduction of phosphorylation (37 phosphopeptides with total dephosphorylation, 94 with decreased phosphorylation), and 6 phosphopeptides with increased phosphorylation and 20 with total phosphorylation (Table S4B). For the comparison between the controls (T0), a total of 3,675 phosphopeptides were identified (location probability >75%), with 157 phosphopeptides significantly differing between $\Delta mpkC$ and WT strains ($P < 0.05$) (Table S5A and B).

In the $\Delta mpkC \Delta sakA$ response to CR for 30 min, 4,855 phosphopeptides were identified (localization probability >75%), and 142 phosphopeptides corresponding to 111 proteins have significant phosphorylation change between mutant and WT (Fig. 4C and Table S6A and B). The double mutant also showed a profile of increased phosphorylation, as observed for the WT and $\Delta sakA$ strains in the CR 30-min response, with 26 phosphopeptides with total dephosphorylation, 12 with decreased phosphorylation, 70 with increased phosphorylation, and 34 with total phosphorylation (Fig. 4C). For the comparison without CR (T0), 3,741 phosphopeptides were observed, with only 54 significantly different between mutant and WT (Table S7A and B).

To determine Gene Ontology (GO) categories overrepresented in the mutant phosphoproteomes, we carried out a functional categorization by FungiFun analysis (Fig. 4D to F). For all mutants, at least 65% of the phosphopeptides have known function and less than 35% are hypothetical proteins (Fig. 4D). For $\Delta sakA$ mutants, most of the proteins with increased phosphorylation upon CR exposure are related to protein binding, epidermal growth factor (EGF) receptor signaling pathway, DNA conformation modification, and DNA or RNA binding. For the group of proteins with decreased phosphorylation upon CR exposure, most of them are protein kinases and proteins related to modification by phosphorylation (phosphorylation, dephosphorylation, or autophosphorylation) (Fig. 4E). For the $\Delta mpkC$ mutant, proteins with increased phosphorylation are mainly related to RNA topology, RNA binding, and rRNA processing, while proteins with decreased phosphorylation are related to cytoskeleton, such as actin cytoskeleton; budding, cell polarity, and filament formation; and spindle pole body/centrosome and microtubule cycle (Fig. 4F). The functional analysis of the double mutant phosphoproteome in CR response by FungiFun did not show enrichment of any pathway (data not shown). However, by using GO enrichment, we were able to identify phosphorylation of proteins related to metabolism, signal transduction, transcription/translation, cell cycle, cytoskeleton, and protein degradation. Enrichment for protein kinases/phosphatases and proteins related to intracellular transport, for example, has

FIG 4 Phosphorylation profile of proteins from *A. fumigatus* and MAPK mutants during incubation with Congo red (CR 10 min). (A to C) Volcano plots of total phosphorylation profile of proteins identified in $\Delta sakA$ mutant versus wild type (WT), $\Delta mpkC$ mutant versus WT, and the double $\Delta sakA \Delta mpkC$ mutant versus WT, respectively. (D) Percentage of proteins with unknown and known function in all three phosphoproteomes $\Delta sakA$ (top); $\Delta mpkC$ (middle); and $\Delta sakA \Delta mpkC$ (bottom). All of them showed more than 60% characterized proteins. (E to G) Functional categorization of approximately 75% of proteins with known function differentially phosphorylated in $\Delta sakA$, $\Delta mpkC$, and $\Delta sakA \Delta mpkC$ strains during CR stress. (H) Venn diagram of total differentially phosphorylated proteins (n = 621) representing the number of phosphoproteins between strains in each intersection (each strain has a set of differentially phosphorylated proteins after CR treatment compared to its untreated control). Thus, the WT strain has a total set of 431 phosphoproteins from which a unique set of 245 is not shared with any other condition, 69 are shared only with the $\Delta sakA$ mutant, 13 are shared only with the $\Delta sakA \Delta mpkC$ mutant, and 38 are shared only with the $\Delta mpkC$ mutant, while 16 phosphoproteins are shared simultaneously by the four strains.

been identified in the double mutant with both increased and decreased phosphorylation (Fig. 4G).

A Venn diagram was generated for correlating the phosphorylation profile between each strain treated with CR (Fig. 4H). Although each strain had a unique set of phosphoproteins (245 for WT, 110 for $\Delta sakA$ mutant, 21 for $\Delta mpkC$ mutant, and 32 for

$\Delta sakA$ $\Delta mpkC$ mutant), among the three mutant strains the $\Delta sakA$ strain showed the highest similarity to the wild-type phosphoproteome during CR exposure, with 69 shared phosphoproteins. The low number of common phosphoproteins among all conditions (16 phosphoproteins) in addition to the low number of common phosphoproteins between the $\Delta mpkC$ and the double $\Delta sakA$ $\Delta mpkC$ mutants (only 2 phosphoproteins) may indicate their independent roles in distinct pathways involved in the CR response.

Interaction networks of identified *A. fumigatus* phosphoproteins. Pairwise analyses identified the differentially phosphorylated proteins after exposure to CR in the different strains. This showed that the phosphoproteomes of wild-type and $\Delta sakA$ strains were functionally enriched for serine/threonine protein kinase domains and RNA recognition motif domains, respectively (see Table S1E for less representative functional enrichments). However, the phosphoproteomes for the $\Delta mpkC$ and the double mutant $\Delta sakA$ $\Delta mpkC$ strains did not have any functional enrichment. The individual sets of differentially phosphorylated proteins in wild-type, $\Delta sakA$, $\Delta mpkC$, and the double mutant $\Delta sakA$ $\Delta mpkC$ strains were combined, yielding 595 proteins which were used to generate a single functional protein association network. The network was composed of 336 nodes and 1862 interactions between nodes (Fig. 5), revealing the enrichment for protein kinases, RNA recognition motif domains, and MAPK signaling pathways (Table S1E). The change of the color pattern in the nodes of Fig. 5A to D highlights the overall network rewiring in terms of differentially phosphorylated proteins for each strain (WT and $\Delta sakA$, $\Delta mpkC$, and $\Delta sakA$ $\Delta mpkC$ mutants) in the presence of CR compared to their respective controls (untreated).

Protein kinases and phosphatases play essential roles in eukaryotic signal transduction pathways. The network analysis showed enrichment for protein kinase and phosphatase domains; hence, a subnetwork highlighting only differentially phosphorylated kinases and phosphatases was extracted (Fig. 6 and Table S1E). The phosphorylation profiles among strains after exposure to CR changed dramatically. In the wild-type strain (Fig. 6A), most of the kinases and phosphatases in the network showed increased phosphorylation compared to the untreated control, including all of the highly connected kinases except for the mitogen-activated protein kinase MpkB (AFUA_6G12820). There was an overall decrease of differentially phosphorylated kinases and phosphatases in $\Delta mpkC$, $\Delta sakA$, and $\Delta sakA$ $\Delta mpkC$ strains in comparison to the wild-type CR response (Fig. 6B to D). In the $\Delta mpkC$ strain, MpkB was the only kinase with increased phosphorylation (Fig. 6B). In contrast to the other strains, the $\Delta sakA$ mutant presented a higher abundance of kinases and phosphatases with decreased phosphorylation after CR exposure (Fig. 6C). The double $\Delta sakA$ $\Delta mpkC$ mutant presented a specific set of differentially phosphorylated proteins, which were not modulated in the same manner in any of the other networks (Fig. 6D). This included the increased phosphorylation of the serine/threonine protein kinase SchA (AFUA_1G06400) and protein phosphatase 2C PtcA (AFUA_1G15800), plus the decreased phosphorylation of the MAP kinase kinase kinase BckA (AFUA_3G11080).

Phosphosite validation. Two approaches were adopted to determine the effects of the identified protein phosphorylation events on *A. fumigatus* CWI: (i) the solved protein structures of available homologues deposited in the Protein Data Bank (PDB) were assessed and (ii) serine/tyrosine residues were mutated to alanine/phenylalanine, or to phosphomimetic aspartate and functionality was assessed. These phosphomutations were introduced by using a CRISPR-Cas9 system. Only a single three-dimensional (3D) structure was available for RckA^{Rck2p}, since no solved structures for the phosphorylated regions of the other chosen proteins were deposited in the PDB. We concentrated our attention on a few proteins, aiming to validate the results obtained by the phosphoproteomics analysis (Fig. 7). Therefore, RckA, MpkB, and SrrB were selected for functional characterization to validate the phosphoproteomics observations.

RckA^{Rck2p} (AFUA_2G03490) is the homologue of the *S. cerevisiae* Rck2p calcium/calmodulin-dependent protein kinase involved in response to oxidative and osmotic

FIG 5 General *A. fumigatus* functional protein association network based on the protein phosphorylation profile during incubation with Congo red (CR 10 min). The whole set of differentially phosphorylated proteins from the wild-type (WT), $\Delta sakA$, $\Delta mpkC$, and $\Delta sakA \Delta mpkC$ strains during CR stress was combined for the generation of a general protein association network. Each node represents a protein that is differentially phosphorylated in at least one strain under CR stress. Node colors are divided into gray nodes, for proteins which were not differentially phosphorylated, and heatmap-colored nodes (blue-white-red), for proteins which were differentially phosphorylated in each strain in relation to its control (the same strain without any stress condition). Node shapes represent molecular functions and are divided into triangles (phosphatases), squares (kinases), and circles (other functions). Each edge represents a functional protein association retrieved from the STRING server (medium confidence threshold of 0.4 for the interaction score), and node sizes represent the degree of each node (number of edges connected to the node). It is important to note that not all the differentially phosphorylated proteins are present in the network, as many proteins did not present any functional associations within the whole set. Changes in the color patterns of nodes from panels A to D represent changes in the differential phosphorylation of the proteins comprising the network in WT, $\Delta sakA$, $\Delta mpkC$, and $\Delta sakA \Delta mpkC$ strains, respectively, during CR stress.

stresses. A single phosphorylation site was identified in RckA (477-MDFRSPGAI-485), which was phosphorylated only when the wild type was exposed to CR (Fig. 7A and B). A BLASTP search performed for *RCK1* homologues with solved structures deposited in the PDB identified a calcium/calmodulin-dependent serine/threonine kinase from

FIG 6 Subnetwork of functional protein associations based only on the phosphorylation profile of protein kinases and phosphatases during incubation with Congo red (CR 10 min). The subset of differentially phosphorylated kinases and phosphatases from the wild-type (WT), $\Delta saka$, $\Delta mpkC$, and $\Delta saka \Delta mpkC$ strains during CR stress was extracted from the general protein association network. Each node represents a protein that was differentially phosphorylated in at least one strain under CR stress. Node colors are divided into gray nodes (proteins that were not differentially phosphorylated) and heatmap-colored nodes (blue-white-red; proteins which were differentially phosphorylated in each strain in relation to its untreated control). Node shapes represent molecular functions and are divided into squares (kinases) and triangles (phosphatases). Each edge represents a functional protein association retrieved from the STRING server (medium confidence threshold of 0.4 for the interaction score). Node size is representative of the number of edges connected to the node. Note that not all the differentially phosphorylated proteins are present in the network, as for many proteins no functional associations were available. Changes in the color patterns of nodes from panels A to D represent changes in the differential phosphorylation of the proteins comprising the network in WT, $\Delta saka$, $\Delta mpkC$, and $\Delta saka \Delta mpkC$ strains, respectively, during CR stress.

Cryptosporidium parvum. The crystal structure of full-length CpCDPK3 (cgd5_820; PDB accession no. 3LIJ) protein in complex with Ca^{2+} and AMP-PNP (adenylyl-imidodiphosphate), at 1.9-Å resolution, was used as a template to build three homology models for RckA with respect to the wild-type S481 residue (corresponding to S309 in

A. **RckA**^{Rck2p}

B.

Site	Mutant	Peptide	Score	Kinase	Fold change (30 min CR)
481	<i>ΔsakA</i>	MDFR S PGAI	0.493	GSK3	Present only in the wild-type

C. **MpkB**^{Fus3p}

D.

Site	Peptide	Score	Predicted Kinase	Fold increase (30 min CR)
183	FMTE Y VATR	0.961	unsp	Present only in <i>ΔmpkC</i>

E. **SrrB**

F.

Site	Peptide	Score	Predicted Kinase	Fold increase (30 min CR)
1466	ARSQ T IGSQ	0.802	PKB	Present in both
		0.511	PKA	
		0.500	CKI	
1469	QTIG S QDGE	0.651	DNAPK	
		0.604	cdc2	
		0.581	ATM	

FIG 7 Sites of phosphorylation (S or T) identified during response to Congo red (CR) in wild-type or three kinase null mutant strains. (A, C, and E) Schematics of the kinases showing their kinase domain (blue or green boxes) and position of phosphorylated amino acid residues highlighted in red. (B, D, and F) Phosphorylation sites identified in the phosphoproteome, according to the conditions indicated. For each residue, a score for a kinase site as predicted by Group-based Prediction System 3.0 (GPS; with medium-stringency cutoff) is indicated.

C. parvum) and the two phosphomutations on the S481 residue (S481A and S481D). The pairwise alignments used for homology modeling were refined by structural inspection to avoid gaps (by deletion or insertion) inside secondary structure elements. Models were analyzed to reveal satisfactory atomic contacts, chemical environment, and stereochemistry as well as high-resolution structures (above 2.0 Å), and they indicate that residue S481 is inserted at the middle of the short loop of the calcium-binding EF-hand motif (Fig. 8A).

The three RckA models with different S481 residues were used in long-trajectory molecular dynamics simulations, to assess differences in structural stability and conformation. RckA models containing S481 and S481A were more stable, while the highest main chain conformational change was observed in the S481D model, whose aspartic residue moves from a buried to a solvent-accessible conformation, thus providing an additional calcium-binding residue. This protein shows different patterns of calcium binding and protein activity from the other two (Fig. 8A).

The *ΔrckA*, RckA^{S481D}, and RckA^{S481A} strains have growth rates and conidiation comparable to the wild-type strain (Fig. 8B). The *ΔrckA* and RckA^{S481A} strains were more sensitive to CR- and CFW-induced cell wall damage, while the RckA^{S481D} strain was

FIG 8 Phosphomutants for RckA serine phosphorylation sites. (A) Molecular modeling for RckA wild type and predicted phosphomutants. (B to F) Growth phenotypes of the wild type and $\Delta rckA$, RckA^{S481D}, and RckA^{S481A} mutants grown for 5 days at 37°C on minimal medium (MM) or MM plus cell wall-damaging or osmotic stress agents (Congo red [CR], calcofluor white [CFW], caspofungin, and sorbitol). (G) Western blot analysis for MpkA phosphorylation in the wild type and $\Delta rckA$, RckA^{S481D}, and RckA^{S481A} mutants grown for 16 h in MM and transferred to MM plus 300 μg/ml of Congo red. Signal intensities were quantified using the Image J software by dividing the intensity of phosphorylated MpkA (MpkA~P) by MpkA.

more resistant to CFW (Fig. 8C and D). Surprisingly, all three RckA mutant strains were more sensitive to caspofungin and completely or partially lost the CPE (Fig. 8E). There were no differences in osmotic stress tolerance among the wild-type, $\Delta rckA$, and RckA^{S481A} strains. However, the RckA^{S481D} strain was more resistant to high sorbitol concentrations than the wild-type or other mutant strains (Fig. 8F). MpkA phosphory-

lation in the $\Delta rckA$ mutant upon exposure to CR was similar to the wild-type strain (Fig. 8G). The $RckA^{S481A}$ mutant had increased MpkA phosphorylation levels that decreased after exposure to CR for 10 and 30 min but increased about twice at 60 min of exposure (Fig. 8G). The $RckA^{S481D}$ mutant has higher levels of MpkA phosphorylation than the wild-type strain upon exposure to CR (Fig. 8G). Taken together, the data suggest that *rckA* mutants impact MpkA phosphorylation upon cell wall damage, strongly suggesting that RckA is important for the CWI pathway.

MpkB (AFUA_6G12820) is the homologue of *S. cerevisiae* Fus3p mitogen-activated serine/threonine protein kinase, involved in mating. A single phosphorylation site was identified in MpkB (179-FMTEYVATR-187) that was phosphorylated only when $\Delta mpkB$ was exposed to CR (Fig. 7C and D). The $\Delta mpkB$ strain has reduced growth and conidiation (28), while the $MpkB^{Y481F}$ mutant has similar growth rates and conidiation as the wild-type strain (Fig. 9A). The $\Delta mpkB$ and $MpkB^{Y183F}$ strains were more sensitive to CR, CFW, caspofungin (also exhibiting reduction in the CPE), and osmotic stress (Fig. 9B). MpkA phosphorylation in the $\Delta mpkB$ mutant was 10-fold less than for the wild type after 10-min exposure to CR (Fig. 9D), while in the $MpkB^{Y183F}$ mutant MpkA phosphorylation was 4-fold higher in untreated cells, but 2-fold lower after 10-min exposure to CR, than in the wild-type strain (Fig. 9D). After many attempts, we were not able to construct an $MpkB^{Y183D}$ mutant.

SrrB (AFUA_2G15010) is a putative response regulator of a two-component phosphorelay system. Two phosphorylation sites (1462-ARSQTIGSQGE-1472) were identified as phosphorylated in both wild-type and $\Delta saka$ strains exposed to CR (Fig. 7E and F). The $SrrB^{S1466-1469A}$ mutant had comparable growth rates and conidiation as the wild-type strain (Fig. 9A). The $SrrB^{S1466-1469A}$ strain was more sensitive to CR, CFW, and caspofungin (with a reduction in the CPE) and had growth comparable to the wild-type strain under high-osmotic-stress conditions (Fig. 9C). MpkA phosphorylation in the $SrrB^{S1466-1469A}$ mutant upon exposure to CR for 10 min was 3-fold less than in the wild-type strain (Fig. 9D).

Taken together, these results suggest that the $RckA^{S481A}$ mutation mimics the loss-of-function mutation, while the $RckA^{S481D}$ mutation allows the mutant strain to be more efficient in coping with cell wall and osmotic stress conditions. However, for all the other phosphomutants there was an increase in the sensitivity to cell wall-damaging agents and a reduction in the MpkA phosphorylation upon CR stress, suggesting these phosphosites could be important for the MpkA modulation and CWI pathway regulation.

DISCUSSION

The filamentous fungal cell wall is essential for survival and serves as a primary means by which fungi interact with their environment. However, relatively limited information is available regarding the major regulatory events involved in cell wall biosynthesis and repair. The *A. fumigatus* module PkcA-MpkA-RlmA is essential for the CWI (36, 41–45). To learn more about the main regulatory posttranslational events that occur during CWI activation, we have investigated the global phosphoproteomics of *A. fumigatus* during cell wall damage caused by CR. A total of 756 high-confidence phosphorylation sites were identified in the *A. fumigatus* wild-type strain. A total of 380 were exclusive to CR-treated samples, and 3 were observed only in the control, while 373 were observed in the presence and absence of CR. These phosphopeptides correspond to 485 proteins. According to FunCat annotation enrichment and the interaction network analyses of this novel phosphoproteome, the proteins phosphorylated in response to CR were involved in a variety of important biological processes, including signal transduction (osmosensing and response, Ca^{2+} -mediated signal transduction, and MAPKKK cascade), stress response, protein kinases, actin cytoskeleton, and budding cell polarity and filament formation. These results emphasize the importance of osmotic stress, calcium metabolism, and MAPKs in the *A. fumigatus* CWI pathway, as previously demonstrated (29, 34, 78–82).

We also investigated the role played by the *A. fumigatus* HOG MAPKs MpkC and

FIG 9 Phosphomutants for MpkB and SrrB serine phosphorylation sites. (A to C) Phenotypes of the wild type and $\Delta mpkB$, MpKBY183F, and SrrBS1466-1469A mutants grown for 5 days at 37°C on minimal medium (MM) or MM plus cell (Continued on next page)

SakA. These two MAPKs are important for the MpkA phosphorylation during cell wall damage (29). Recently, SakA was demonstrated to physically associate with MpkC upon osmotic and CR-induced cell wall stresses (34). We were not able to identify a phosphorylation pattern that explains how MpkC modulates SakA function. Under cell wall stress, SakA also physically interacts with the CWI MAPK, MpkA, and the PtcB MAPK phosphatase, suggesting a close connection between the HOG and CWI pathways. Cross-talk between the HOG and CWI pathways has been observed in several fungal species, including *S. cerevisiae*, *Candida albicans*, *Fusarium graminearum*, and *Pyrenophora graminea* (56–64). Now, we show that the absence of these two HOG pathway-associated MAPKs, MpkC and SakA, abolishes the remodeling of the phosphoproteome seen in the wild-type fungus in response to cell wall damage, and we also reveal novel proteins potentially involved in cell wall stress response.

Our global phosphoproteome network analysis showed an enrichment for protein kinases, RNA recognition motif domains, and the MAPK signaling pathway. In contrast to the wild-type strain, there was an overall decrease of differentially phosphorylated kinases and phosphatases, among them RckA^{Rck2p}, MpkB^{Fus3p}, and SrrB, in $\Delta mpkC$, $\Delta sakA$, and $\Delta sakA \Delta mpkC$ strains. We introduced phosphomutations in these proteins and assessed their tolerance of cell wall-damaging agents and osmotic stress conditions. Although it is very difficult to assign a single role for a specific protein kinase or phosphatase in the CWI pathway, the validation of our results by introducing corresponding phosphomutations in two protein kinases (RckA and MpkB) and a putative response regulator of a two-component phosphorelay system (SrrB) strongly indicated the importance of the osmotic stress and the cross-talk among HOG and MpkB^{Fus3p} pathways in the CWI pathway. This is reflected in the fact that most of the *A. fumigatus* putative homologues of the components of the three MAPK pathways were differentially phosphorylated or dephosphorylated in the presence or absence of cell wall damage (Fig. 10).

We discovered several protein kinases and TFs involved and validated their importance in the *A. fumigatus* cell wall damage response. None of the 11 kinase null mutants were found to be more sensitive or resistant to several cell wall or osmotic stresses. Hence, further experimental validation will help to determine which kinases are important to the osmotic stress and cell wall damage response pathways. In contrast, all five TF null mutants showed cell wall stress-related phenotypes. This included the AreB (AFUB_029020) GATA zinc finger transcription factor, which negatively regulates nitrogen catabolism (65). The *A. nidulans areB* null mutant has significant phenotypic effects on the utilization of specific carbon sources, confirming its role in the regulation of carbon metabolism (66). Considering the importance of carbon source assimilation in the remodeling of the cell wall could explain why in *A. fumigatus* AreB is important for the cell wall damage response. Another TF was AsgA (AFUB_019790), the homologue of *S. cerevisiae* Asg1p, a zinc cluster protein proposed to be a transcriptional regulator involved in the stress response. The *S. cerevisiae* $\Delta asg1$ mutant has a respiratory defect and is sensitive to CFW (67). It is possible that *A. fumigatus* AsgA is similarly involved in some aspects of the cell wall damage response. In *S. cerevisiae*, the forkhead family transcription factor Fkh2 is involved in the activation of early-firing replication (68) and Fkh2 mutations suppress the checkpoint-defective phenotype of both HOG and cell wall integrity MAPK mutations (63). Therefore, the *A. fumigatus* homologue, FkhA, could be a putative CWI checkpoint for cell cycle progression in *A. fumigatus*.

Our global phosphoproteome analysis of the fungal response to cell wall damage has provided the identification of several novel proteins that are important for cell wall stress tolerance. These results will provide opportunities to understand how the signal

FIG 9 Legend (Continued)

wall-damaging or osmotic stress agents (Congo red [CR], calcofluor white [CFW], caspofungin, and sorbitol). (D) Western blot analysis for MpkA phosphorylation in wild type and $\Delta mpkB$, MpkB^{Y183F}, and SrrB^{S1466–1469A} mutants grown for 16 h in MM and transferred to MM plus 300 μ g/ml of Congo red. Signal intensities were quantified using the Image J software by dividing the intensity of MpkA~P by γ -tubulin.

FIG 10 Differential phosphorylation of MAPK pathways during response to cell wall damage. The wild-type, $\Delta sakA$, $\Delta mpkC$, and $\Delta sakA \Delta mpkC$ strains were exposed to CR for 30 min. Phosphorylated (green boxes) or dephosphorylated (red boxes) proteins in the presence of CR are shown in the different MAPK pathways. Superscript names correspond to the respective homologue genes in *S. cerevisiae*. Proteins with no phosphorylation modulation are represented with the *S. cerevisiae* corresponding name, adapted from the MAPK signaling pathway, yeast, *Aspergillus fumigatus*, in KEGG (Kyoto Encyclopedia of Genes and Genomes [<https://www.genome.jp/kegg/>]).

transduction networks that modulate the integrity of the cell wall function. In addition, our results highlight potential new targets for interference in fungal signaling events and cell wall remodeling to promote the efficacy of existing antifungals while impeding virulence.

MATERIALS AND METHODS

Strains and growth medium. *Aspergillus fumigatus* strain CEA17 was used for CRISPR-Cas9 editing (Table 2). Fungal strains were cultivated in liquid YPD medium (1% yeast extract, 1% polypeptone, and 1% glucose) or in solid minimal medium (MM): 1% glucose, nitrate salt solution (1×), trace metal solution (1×), 0.001% thiamine, and 2% agar (69, 70). For transformation, TM medium (transformation medium; with 1 M sucrose instead of 1% glucose) was used. After the confirmation of the mutations by DNA sequencing, fungal strains were grown in MM supplemented with 10 mM uridine (Uri) and 10 mM uracil (Ura) and 5-fluoroorotic acid (5-FOA) (71). *Escherichia coli* DH5α was used for amplification of CRISPR vectors and was grown in solid or liquid LB medium containing 100 μg/ml ampicillin.

Fungal growth, protein extraction, digestion, and phosphoenrichment. *A. fumigatus* conidia (1×10^7) from the wild-type (WT) and mutant strains ($\Delta sakA$, $\Delta mpkC$, and $\Delta sakA \Delta mpkC$ strains [Table 2]) (72) were inoculated in YG medium {0.5% yeast extract, 1% dextrose; 0.1% trace elements [22.0 g/liter $ZnSO_4$, 11 g/liter boric acid, 5 g/liter $MnCl_2$, 5 g/liter $FeSO_4$, 1.6 g/liter $CoCl_2$, 1.6 g/liter $CuSO_4$, 1.1 g/liter $(NH_4)_2MoO_4$]} and incubated for 16 h under 200-rpm rotation and 37°C. After that, 300 μg/ml of Congo red (CR) was added, followed by incubation for 10 min (or time zero, as control [T0]). Mycelia were then filtered using a vacuum system and frozen by liquid nitrogen. Frozen mycelia were macerated, and protein was extracted by the addition of TNE buffer (50 mM Tris-HCl [pH 7.5], 140 mM NaCl, 5 mM EDTA, phosphatase inhibitor cocktail [PhosphoStop; Roche], EDTA-free protease inhibitor cocktail [Roche], and 0.1 mM phenylmethylsulfonyl fluoride [PMSF]) and incubation for 15 min under agitation following centrifugation at $10,000 \times g$ for 20 min. The supernatant was collected, and total protein was quantified by the Bradford assay. Five hundred micrograms of protein was purified by 3-kDa-molecular-weight-

TABLE 2 Strains used in this work

Name	Genotype	Source or reference
CEA17	Wild-type strain	FGSC A1159
$\Delta sakA$	$\Delta sakA::hph$	29
$\Delta mpkC$	$\Delta mpkC::ptrA$	29
$\Delta sakA \Delta mpkC$	$\Delta sakA::hph \Delta mpkC::ptrA$	29
$\Delta mpkB$	$\Delta mpkB::ptrA$	28
MFIG001	$\Delta akuB pyrG^+$	FGSC A1160
$\Delta hriA$ (AFUB_032300)	$\Delta hriA::hph \Delta akuB$	This study
$\Delta srrB$ (AFUB_030660)	$\Delta srrB::hph \Delta akuB$	This study
$\Delta kin1$ (AFUB_010510)	$\Delta kin1::hph \Delta akuB$	This study
$\Delta snfA$ (AFUB_018770)	$\Delta snfA::hph \Delta akuB$	This study
$\Delta cmkA$ (AFUB_029320)	$\Delta cmkA::hph \Delta akuB$	This study
$\Delta prr1$ (AFUB_029820)	$\Delta prr1::hph \Delta akuB$	This study
$\Delta pkhA$ (AFUB_036500)	$\Delta pkhA::hph \Delta akuB$	This study
$\Delta cmkC$ (AFUB_053520)	$\Delta cmkC::hph \Delta akuB$	This study
$\Delta kfsA$ (AFUB_014350)	$\Delta kfsA::hph \Delta akuB$	This study
$\Delta oca2$ (AFUB_059390)	$\Delta oca2::hph \Delta akuB$	This study
$\Delta fpkA$ (AFUB_059090)	$\Delta fpkA::hph \Delta akuB$	This study
$\Delta AFUB\_004210$	$\Delta AFUB\_004210 \Delta akuB$	This study
$\Delta AFUB\_009270$	$\Delta AFUB\_009270 \Delta akuB$	This study
$\Delta asgA$ (AFUB_019790)	$\Delta asgA::hph \Delta akuB$	This study
$\Delta areB$ (AFUB_029020)	$\Delta areB::hph \Delta akuB$	This study
$\Delta fkhA$ (AFUB_037220)	$\Delta fkhA::hph \Delta akuB$	This study
Afu6g12820_ mpkB _184Phe	Y184P mutation	This study
Afu2g15010_ srrB _1466Ala1469Ala	S1466A and S1469A mutations	This study
Afu2g15010_ srrB _1466Asp1469Asp	S1466D and S1469D mutations	This study

cutoff ultrafiltration (Centriprep YM-3; Millipore, Billerica, MA) followed by trichloroacetic acid (TCA) precipitation. TCA was added to protein extract at a 10% (wt/vol) final concentration, vortexed for 15 s, and placed on ice for a minimum of 30 min. Sample were centrifuged at $14,000 \times g$ for 15 min, and the supernatant was discarded. The pellet was washed three times with cold acetone with centrifugation at $14,000 \times g$, 4°C, for 10 min. Protein pellets were reduced by the addition of dithiothreitol (DTT) and alkylated by the addition of iodoacetamide. Protein digestion was performed by the addition of sequencing-grade trypsin (Promega) (1:50) for 16 h at 37°C. Phosphopeptides were enriched with the Pierce TiO₂ phosphopeptide enrichment and cleanup kit (Thermo Fisher Scientific) according to the manufacturer's instructions. The strains used in this paper are listed in Table 2.

LC-MS/MS. Phosphopeptide identification and analysis were performed with at least 4 replicates on a nano-liquid chromatograph (nanoLC) (NanoAcquity; Waters Corporation, Milford, MA) coupled to an Orbitrap Fusion Tribrid mass spectrometer (Thermo Fisher, San Jose, CA). Samples were resolved in 10 μ l of acetonitrile (ACN)-H₂O-formic acid (FA) (5/95/0.1, vol/vol/vol) for LC-MS/MS analysis. For each run, 2 μ l of sample was loaded on a 100- μ m (inner diameter [i.d.]) by 20-mm, 5- μ m C₁₈ precolumn at a flow rate of 4 μ l/min for 5 min, using a loading buffer of ACN-H₂O-FA (5/95/0.1, vol/vol/vol). Peptide separation was performed on a 75- μ m (i.d.) by 180-mm, 5- μ m C₁₈ analytical column at a flow rate of 250 nl/min in a 95-min gradient by using mobile phase A of 0.1% formic acid in water and mobile phase B of 0.1% FA in acetonitrile. The gradient elution started at 5% mobile phase B, was increased to 35% at 60 min and 80% at 65 min, and was held at 80% for 5 min before a 25-min reequilibration at 5%. The eluting peptides were interrogated with an Orbitrap Fusion mass spectrometer running a data-dependent LC-MS/MS method with the Top Speed decision selection. FTMS1 (Fourier Transform Mass Spectrometry) spectra were collected using the following parameters: scan range 350 to 1,800 m/z , resolving power 120k, automatic gain control (AGC) target 4E5, and maximum injection time of 50 ms. ITMS2 (Ion Trap Tandem Mass Spectrometry) spectra were collected using the following parameters: rapid scan rate, collision-induced dissociation (CID) NCE (normalized collision energy) 35, 1.6 m/z isolation window, AGC target 1E4, and maximum injection time of 50 ms. MS2 (Mass Spectrometry) precursors were selected for a 3-s cycle. Precursors with an assigned monoisotopic m/z and a charge state of 2 to 7 were interrogated. Precursors were filtered using a 60-s dynamic exclusion window.

Phosphopeptide identification and protein analysis. Raw mass spectrometric data were analyzed in the MaxQuant environment (version 1.6.0.16). The MS/MS spectra were matched against the *Aspergillus fumigatus* UniProt FASTA database. Enzyme specificity was set to trypsin, and the search included cysteine carbamidomethylation as a fixed modification and N-acetylation of protein, oxidation of methionine, and/or phosphorylation of Ser, Thr, and Tyr residues (STY) as variable modifications. Up to two missed cleavages were allowed for protease digestion, and peptides had to be fully tryptic.

The analyses of phosphopeptide, phosphorylation sites, and number of phosphoresidues were performed using filter tools in the software Microsoft Office Excel. Student's *t* test (two-tailed, 95% [$P < 0.05$]) was performed for identification of differentially phosphorylated peptides between CR and T0 wild-type samples, CR mutant and CR WT samples, or T0 mutant and T0 wild-type samples. The total phosphopeptides identified were used for kinase site prediction by the software GPS 3.0, according to the default parameters, including medium threshold. For analysis of conservation between kinase family

phosphosites, all sequences obtained from GPS software were selected for sequence logo building for the p38 MAPK family, using the software Weblogo (<http://weblogo.berkeley.edu/logo.cgi>), according to default parameters. All heatmaps were produced using Permutmatrix software (version 1.9.3.EN; <http://www.atgc-montpellier.fr/permutmatrix/>) according to default parameters, including Euclidean distances. Protein categorizations were performed by the FungiFun website (<https://sbi.hki-jena.de/fungifun/>) using *A. fumigatus* Af293 as species and identifiers (IDs) from each supplemental table (only "AFUA_" IDs were used).

Vector construction for CRISPR-Cas9. Vector construction was performed according to the methods described in reference 73. Primers and donor oligonucleotides (TAG Copenhagen, Copenhagen, Denmark) were designed using the *A. fumigatus* genomic sequence of each target (Afu6g12820_mpkB, Afu2g15010_srrB, and Afu6g04500_amk2; available on the Aspergillus Genome Database [AspGD], <http://www.aspergillusgenome.org/>) and are indicated in Table S8A and B in the supplemental material.

The single guide RNAs (sgRNAs) were linked into the CRISPR-Cas9 vector pFC330 according to the tails of two primers. Previously, PCR fragments were amplified using the *Pfu* X7 polymerase with the primers from Table S8A and using pFC902 as a template. The "biobrick" fragments were further fused to the digested pFC330 by uracil-specific excision reagent (USER) fusion and USER cloning (74). Plasmids were purified with the GenElute plasmid miniprep kit (Sigma-Aldrich) and confirmed by restriction analysis. The selection of the transformants was performed using MM as selective medium, because the CRISPR-Cas9 vector (pFC330) contains *pyrG* as an auxotrophic marker.

Protoplastization and genetic transformation. Protoplastization of *A. fumigatus* was achieved using protocols previously described (73, 75). For transformation, 5 to 10 μ l of the 100 μ M stock donor oligonucleotide solution (Table S2) and 10 μ g of the CRISPR-Cas9 vector were mixed with 50 μ l protoplasts, 150 μ l of PCT solution (50% [wt/vol] polyethylene glycol [PEG] 8000, 50 mM CaCl_2 , 20 mM Tris, 0.6 M KCl, pH 7.5) was added, and the sample was incubated at room temperature for 10 min. The mixture was then mixed with 250 μ l ATB solution (1.2 M sorbitol, 50 mM $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$, 20 mM Tris, 0.6 M KCl, pH 7.2). The protoplasts were plated on osmotic and selective TM medium and incubated at 37°C until transformants appeared on the transformation plates (approximately 1 week). Transformants were restreaked four times in MM, genomic DNA was extracted, and the PCR fragments for each gene were sent for DNA sequencing to confirm the presence of the respective mutation (the list of primers used for sequencing is in Table S8A).

Protein-protein network analysis. For network analysis, phosphoproteomic data were filtered for differences in phosphorylation level of >2-fold or <-2-fold and *P* value of >0.05. Next, data were filtered and analyzed through ad hoc R scripts (total number of differentially expressed phosphoproteins = 621) for the generation of Venn diagrams (VennDiagram package obtained from the following CRAN: <https://cran.r-project.org/web/packages/VennDiagram/index.html>) representing the intersections of the differentially phosphorylated proteins between the previously described studied strains. The list of differentially phosphorylated proteins and functional enrichment was obtained using the STRING server (<https://string-db.org/>), medium confidence threshold of 0.4 for interaction scores, using *Aspergillus fumigatus* as a model organism, and the generated functional protein association network presented a total of 336 nodes and 1,862 interactions as 26 phosphoproteins could not be found on the STRING server and the other 259 phosphoproteins did not present any association scores within the given set. The association network was then reprocessed through ad hoc R scripts for acquiring only "AFUA_" IDs for each of its nodes and was finally plotted/analyzed in Cytoscape software (<https://cytoscape.org/>). For network visualization, the fold change of phosphorylation levels was transformed to $\log_2$ and loaded in Cytoscape for color mapping as a heatmap scale (blue-white-red), and the node degree was mapped to node size. Node shapes were also mapped to protein functional domains representing three classes: kinases, phosphatases, and other protein domains.

Sequence alignment and homology modeling procedures. The search for *RCK1* homologues with solved structures deposited in the Protein Data Bank (PDB; <http://www.rcsb.org>) was performed using the web server BLASTp (Basic Local Alignment Search Tool, for proteins) algorithm (<https://blast.ncbi.nlm.nih.gov/Blast.cgi?PAGE=Proteins>). A protein with the PDB ID 3LUJ (crystal structure of full-length CpCDPK3 [cgd5_820] in complex with Ca^{2+} and AMP-PNP, at 1.9-Å resolution—a calcium/calmodulin-dependent serine/threonine kinase from *Cryptosporidium parvum*) was detected and here used as a template to build three homology models for *RCK1*, with respect to the wild-type residue (S481) and two mutations on residue 481 (S481A and S481D), using Modeller v. 9.2 (76). The pairwise alignments used as input for Modeller were previously performed using the web server Clustal Omega (v. 1.2.4; <https://www.ebi.ac.uk/Tools/msa/clustalo/>) and subsequently refined by structural inspection of the template, thus avoiding gaps (by deletion or insertion) inside secondary structure elements. Models were analyzed/validated regarding atomic contacts, chemical environment, and stereochemistry, using the web server SAVES v.5.0, at <http://servicesn.mbi.ucla.edu/SAVES/>.

MD procedures. Molecular dynamics (MD) calculations were carried out using the Biovia Discovery Studio software (77), founded on the CHARMM molecular mechanics force field engine. We used the standard dynamics cascade. Molecular dynamics was done with no restraints using as input the homology models built for *RCK1* (Afu2g03490), a calcium/calmodulin-dependent protein kinase from *A. fumigatus*, with respect to two mutations on residue S481 (S481A and S481D). At the first step, energy minimization was carried out using steepest descent with 100 maximum steps and a root mean square (RMS) gradient of 1.0. At the second step of the MD protocol, energy minimization was performed setting the conjugated gradient as algorithm, with 500 maximum steps and an RMS gradient of 0.1. For the heating step, we used a simulation time of 50 ps, with 2-ps time steps and initial and target temperatures of 50 and 300 K, respectively. The equilibration phase was carried out with 100 ps and target temperature

of 300 K. On the production step, a simulation time of 1,000,000 ps (1,000 ns) was considered, saving results at 1,000-ps intervals (snapshots). We used an implicit solvent dielectric constant of 80 (simulation in water), with a nonbond higher cutoff distance of 12.0 and nonbond lower cutoff distance of 10.0, with a leapfrog Verlet dynamics integrator.

Phenotypic characterization of *A. fumigatus* null mutant strains. *A. fumigatus* conidia (10^5) of wild-type (CEA17) and null mutant strains (Table 2) were inoculated on solid minimal medium (MM; 1% glucose, 6 g/liter NaNO_3 , 0.52 g/liter KCl, 1.52 g/liter KH_2PO_4 , 0.52 g/liter $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 1 ml/liter of trace elements, pH 6.5, 2% agar) with different concentration of CR, CFW, sorbitol, and caspofungin and grown for 5 days at 37°C. The radial growth of each strain in the presence of the molecules was normalized by the growth of each strain in MM without any molecule. All plates were grown in triplicate, and the average $\pm$ SD was plotted. A Student *t* test using Prism GraphPad (version 6) was applied to confirm the statistical significance difference between treatment and control (*P* value < 0.05).

SUPPLEMENTAL MATERIAL

Supplemental material is available online only.

FIG S1, PDF file, 0.1 MB.

FIG S2, PDF file, 0.03 MB.

TABLE S1, XLSX file, 2.5 MB.

TABLE S2, XLSX file, 2.7 MB.

TABLE S3, XLSX file, 2 MB.

TABLE S4, XLSX file, 2.6 MB.

TABLE S5, XLSX file, 2.2 MB.

TABLE S6, XLSX file, 2.7 MB.

TABLE S7, XLSX file, 2.1 MB.

TABLE S8, DOCX file, 0.01 MB.

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G.H.G. and E.C.M. conceived the study, and each designed and supervised substantial parts of it. E.C.M., L.P.S., C.V., P.A.C., T.F.D.R., L.F.C.R., R.S.-R., C.W., C.H.T.D.P.D.S., and C.A.T. performed the experiments and acquired the data. M.B. and N.A.F. provided some of the *A. fumigatus* deletion strains. All authors helped to analyze and interpret the data and read and approved the final manuscript. G.H.G. and E.C.M. drafted the manuscript, which was critically revised by M.R.M., M.B., U.H.M., J.P.B., and N.A.B.

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Aspergillus fumigatus High Osmolarity Glycerol Mitogen Activated Protein Kinases SakA and MpkC Physically Interact During Osmotic and Cell Wall Stresses

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Aspergillus fumigatus, a saprophytic filamentous fungus, is a serious opportunistic pathogen of mammals and it is the primary causal agent of invasive aspergillosis (IA). Mitogen activated protein Kinases (MAPKs) are important components involved in diverse cellular processes in eukaryotes. *A. fumigatus* MpkC and SakA, the homologs of the *Saccharomyces cerevisiae* Hog1 are important to adaptations to oxidative and osmotic stresses, heat shock, cell wall damage, macrophage recognition, and full virulence. We performed protein pull-down experiments aiming to identify interaction partners of SakA and MpkC by mass spectrometry analysis. In presence of osmotic stress with sorbitol, 118, and 213 proteins were detected as possible protein interactors of SakA and MpkC, respectively. Under cell wall stress caused by congo red, 420 and 299 proteins were detected interacting with SakA and MpkC, respectively. Interestingly, a group of 78 and 256 proteins were common to both interactome analysis. Co-immunoprecipitation (Co-IP) experiments showed that SakA::GFP is physically associated with MpkC:3xHA upon osmotic and cell wall stresses. We also validated the association between SakA:GFP and the cell wall integrity MAPK MpkA:3xHA and the phosphatase PtcB:3xHA, under cell wall stress. We further characterized *A. fumigatus* PakA, the homolog of the *S. cerevisiae* sexual developmental serine/threonine kinase Ste20, as a component of the SakA/MpkC MAPK pathway. The $\Delta pakA$ strain is more sensitive to cell wall damaging agents as congo red, calcofluor white, and caspofungin. Together, our data supporting the hypothesis that SakA and MpkC are part of an osmotic and general signal pathways involved in regulation of the response to the cell wall damage, oxidative stress, drug resistance, and establishment of infection. This manuscript describes an important biological resource to understand SakA and MpkC protein interactions. Further investigation of the biological roles played by these protein interactors will provide more opportunities to understand and combat IA.

Keywords: *Aspergillus fumigatus*, mitogen activate protein kinase, HOG, MPKC, SakA

INTRODUCTION

Protein-protein interactions (PPI) are essential for the accomplishment of many biological functions (Bork et al., 2004). Interactome analyses can increase the understanding of the biological interactions and cellular processes within an organism. Recent high-throughput studies have obtained a great amount of PPI data, including model organisms such as *Saccharomyces cerevisiae* (Lam et al., 2015) and *Aspergillus nidulans* (Bayram et al., 2012; Jaimes-Arroyo et al., 2015), as well as plant pathogens *Fusarium graminearum* (Zhao et al., 2009), *Magnaporthe grisea* (He et al., 2008), and *Phomopsis longicolla* (Li et al., 2018). Bacteria, plants (Nishiyama et al., 2013), and fungi (Aguirre et al., 2006) use conserved phosphorelay systems to sense different types of environmental stresses. Mitogen activated protein Kinases (MAPKs) are important components involved in diverse cellular processes in eukaryotes (Pearson et al., 2001). Filamentous fungi contain homologous MAPKs which mediate specific cell signaling events and coordinated the appropriate biological response. MAPK pathways control the response to multiple stresses including, oxidative, osmotic, heat shock, reactive oxygen species, nutrient limitation, and high concentrations of heavy metals (Risipail et al., 2009; Hamel et al., 2012; Turra et al., 2014).

In *S. cerevisiae*, MAPK Hog1p is involved in many aspects of the osmotic stress response, such as ion transport across cell membranes, cell cycle progression, and regulation of transcription and translation processes (Martínez-Montañés et al., 2010; de Nadal and Posas, 2015). In *A. nidulans*, a conserved phosphorelay cascade activates the MAPK SakA/HogA (Vargas-Perez et al., 2007) in response to oxidative, osmotic, and nutrient starvation stresses (Kawasaki et al., 2002; Lara-Rojas et al., 2011). SakA is a *S. cerevisiae* Hog1p homolog and in association with the AtfA transcription factor was shown to be involved in osmotic stress response (Hagiwara et al., 2009). In addition, SakA phosphorylation is required for asexual and sexual development (Lara-Rojas et al., 2011). During oxidative stress, SakA interacts with AtfA, which then activates the catalase genes *catA* and *catB* (Lara-Rojas et al., 2011). The *sakA* and *atfA* deletion mutants are sensitive to oxidative stress. In other filamentous fungi the SakA/Hog1p orthologs have been shown to have roles in osmotic and oxidative stress responses, while also being involved the regulation of development, and/or virulence (Eaton et al., 2008; Heller et al., 2012; Lamb et al., 2012; Van Nguyen et al., 2013; Nimmanee et al., 2015).

Aspergillus fumigatus is a saprophytic filamentous fungus and a deadly opportunistic pathogen of mammals (Greenberger, 2002; Dagenais and Keller, 2009). It is the primary causal agent of Invasive Aspergillosis (IA), one of the most common life threatening fungal diseases in neutropenic patients and has been shown to have mortality rates that can reach 90% (Brakhage, 2005; Brown et al., 2012a,b; Lackner and Lass-Flörl, 2013). It has been reported that several phenotypes influence the final outcome of the IA establishment showing that aspergillosis is a multifactorial disease (Tekaiia and Latgé, 2005; Hartmann et al., 2011; Sugui et al., 2014). The signaling pathways that regulate these factors involved in virulence are essentials for *A. fumigatus*

survival within the human host (Brown and Goldman, 2016). As many filamentous fungi, *A. fumigatus* has also four different MAPKs named as MpkA, MpkB, MpkC, and SakA. The MpkA function is mainly related to cell wall integrity (CWI, Valiante et al., 2015a). The MpkB, which is homologous to yeast Fus3, has not been yet characterized (Elion et al., 1990). In *A. fumigatus*, MpkC and SakA are homologs of the *S. cerevisiae* Hog1 and are the major regulators of the osmotic stress response (Risipail et al., 2009). MpkC and SakA also play a role in carbon source utilization and caspofungin adaptation, respectively (Reyes et al., 2006; Altwasser et al., 2015; Valiante et al., 2015b).

Our group recently showed that MpkC and SakA are important to adaptations to oxidative and osmotic stresses, heat shock, and cell wall damage (de Oliveira Bruder Nascimento et al., 2016). The double mutant $\Delta mpkC \Delta sakA$ demonstrated increased sensitivity to the above mentioned stresses when compared to the $\Delta sakA$ and $\Delta mpkC$ single mutants. In addition, this interaction was crucial for macrophage recognition and full virulence. In most of stress conditions tested, the phenotypes of $\Delta sakA$ were intensified by the $\Delta mpkC$ mutation, while $\Delta mpkC$ phenotypes were moderate (de Oliveira Bruder Nascimento et al., 2016). Accordingly, we proposed that SakA and MpkC are interactive and that MpkC could be a modulator of SakA during HOG and CWI pathways (de Oliveira Bruder Nascimento et al., 2016).

No information is available concerning the SakA and MpkC protein targets during osmotic and cell wall stresses, and very little is known about the mechanisms by which SakA and MpkC control these stress responses. Our aim was to identify SakA and MpkC targets that could mediate their functions. We demonstrate that SakA and MpkC show functional and physical interactions and that these MAPKs of the HOG pathway play important roles in the CWI pathway. We report that the identified PakA kinase, similar to Ste20, a HOG pathway protein in *S. cerevisiae*, and the MpkA central regulator of CWI pathway, associate with SakA/MpkC MAPK pathway. Finally, we show that during cell wall stress, SakA associates with PtcB, a HOG response phosphatase, involved in regulation of MpkA and SakA phosphorylation. This manuscript provides an important resource to understand SakA and MpkC protein interactions in response to osmotic stress and cell wall damage. Further investigation of the biological roles played by these protein interactors will provide more opportunities to investigate and combat IA.

RESULTS

Identification of Proteins That Interact With SakA and MpkC During Osmotic and Cell Wall Stresses

In *A. fumigatus* SakA and its paralog MpkC, are involved in osmotic stress, nitrogen and carbon starvation, caspofungin tolerance and are important to adaptations to oxidative stress, heat shock, and cell wall damage (Reyes et al., 2006; Risipail et al., 2009; Altwasser et al., 2015; Valiante et al., 2015a;

de Oliveira Bruder Nascimento et al., 2016). In an attempt to further explore the regulatory processes and diverse functions of SakA and MpkC, we sought to identify their interacting protein partners. As previously shown the SakA:GFP and MpkC:GFP strains are functional (de Oliveira Bruder Nascimento et al., 2016). We performed protein pull-down experiments aiming to identify interaction partners of SakA and MpkC by mass spectrometry assays. Protein extracts were prepared from wild-type, SakA:GFP and MpkC:GFP cultures grown for 24 h and further exposed to sorbitol 1.0 M (10, 30, and 60 min) or CR 300 µg/ml (5, 15, 30, and 60 min). We compared proteins that immunoprecipitated with the SakA:GFP or MpkC:GFP and wild-type negative control, removing proteins that precipitated with the latter because they are potential artifacts (Supplementary Tables S1–S8). The full group of proteins that co-purified with SakA and MpkC during osmotic and CR stresses are listed in Supplementary Tables S1, S2, respectively.

In presence of sorbitol, 118 and 213 proteins were detected as possible protein interactors of SakA and MpkC, respectively.

Interestingly, a group of 78 proteins were common to both protein interactomes (Figure 1A). Under CR stress, 420 and 299 proteins were detected interacting with SakA and MpkC, respectively (Figure 1B). Once more a common group of 256 proteins were identified in both interactomes (Figure 1B) which strongly suggests that SakA and MpkC have some common functions in *A. fumigatus*. Among the proteins considered associated with MpkC (Afu5g09100), SakA (Afu1g12940) was identified under CR and osmotic stresses (Supplementary Tables S9, S10), suggesting that MpkC is a SakA interactor. To verify possible interaction networks, the proteins identified by proteomics were analyzed using STRING¹. We were able to identify about 72.0–87.8% of the immunoprecipitated proteins as biologically interacting with either MpkC:GFP, SakA:GFP, both proteins or proteins that interact with them (Supplementary Tables S11, S12; osmotic and CR: MpkC:GFP, 80.3, and 85.3%; SakA:GFP, 72.0 and 84.3%; and MpkC:GFP and SakA:GFP, 79.2 and 87.8%).

¹<https://string-db.org/>

FunCat² enrichment analyses for both strains under osmotic stress demonstrated an enrichment for proteins involved in phosphate metabolism, protein processing, ATP and GTP binding, and translation (osmotic stress, **Figure 1C**). FunCat for both strains upon CR stress showed an enrichment for proteins involved in unfolded protein response, translation, stress response, protein processing, electron transport, biosynthesis of glycine and arginine, and amino acid metabolism (**Figure 1D**). This implies that SakA and MpkC collaborate in several biological processes involving stress responses, translation and protein modification, and amino acid metabolism upon osmotic and cell wall stresses.

SakA and MpkC Interact to Regulate Cell Membrane and Wall Biogenesis During Stress

Among the proteins identified in the SakA or MpkC interactome there are potential partners that can be associated to the functions of these kinases. These comprise MpkA (Afu4g13720, **Tables 2, 4**), the central regulator of CWI pathway, whose phosphorylation during cell wall, and osmotic stresses is regulated by SakA (de Oliveira Bruder Nascimento et al., 2016), and PtcB (Afu1g09280, **Table 3**), a putative HOG phosphatase involved in regulation of MpkA and SakA phosphorylation (Bom et al., 2015). PakA (Afu2g04680, **Table 3**), found to be associated with SakA, is the putative homolog of *S. cerevisiae* Ste20, which is involved in the response to osmotic stress (Tanaka et al., 2014). Other proteins that co-purified with SakA or MpkC include some proteins involved in the biosynthesis of the cell wall polysaccharides (Afu3g12690, Afu7g02180, Afu6g12400, Afu1g06210, Afu2g05340, Afu7g05450, and Afu3g14420, **Tables 1–4**) and ergosterol (Afu3g10660, Afu6g14200, Afu5g02450, Afu7g03740, Afu4g06890, Afu4g07130, and Afu4g03630, **Tables 1–3**). The transcription factors DvrA (Afu3g09820, **Table 1**), the putative *C. albicans* Bcr1p ortholog, that regulates biofilm formation and expression of cell-surface genes, and NsdD (Afu3g13870, **Tables 1, 3, 4**), required during an early stage of mating, that plays a role in resistance toward cell wall stress, were also identified in the SakA interactome. In response to osmotic and cell wall stresses, SakA and MpkC were also found to be associated with some heat shock proteins and chaperones (Afu2g02320, Afu7g01860, Afu1g06710, Afu1g01740, Afu6g07540, Afu7g01860, Afu3g14540, Afu5g13920, and Afu2g16020, **Tables 1–4**). We observed the interaction of these MAPK with the glucan synthase Fks1 (Afu6g12400), which facilitates the production of the major cell wall component 1,3 β -D-glucan, Agm1 (Afu1g06210), a N-acetylphosphoglucosamine mutase involved in chitin biosynthesis, Gel4 (Afu2g05340), a essential 1,3 β -glucanosyltransferase and Sun1 (Afu7g05450), a 1,3 β -glucan modifying enzyme involved in fungal morphogenesis (**Tables 1–4**). SakA and MpkC also interact with proteins involved in the ergosterol biosynthetic pathway including the HMG-CoA synthase Erg13 (Afu3g10660) and the sterol

demethylase proteins, Cyp51A (Afu4g06890) and Cyp51B (Afu7g03740), that are related to the mechanisms for azole drug resistance (**Tables 1–4**). These proteins are possibly related to SakA and MpkC functions.

In summary, SakA and MpkC show both physical and functional interactions and that SakA/MpkC pathways play important roles in the signaling processes that regulate the response to osmotic stress and cell wall damage.

SakA and MpkC Physically Associate During Osmotic and Cell Wall Stresses

To confirm the physical association between SakA and MpkC we decide to carry out co-immunoprecipitation (Co-IP) experiments (see section “Materials and Methods”) using protein extracts from wild-type and strains expressing SakA:GFP and SakA:GFP MpkC:3xHA. We observed no changes in the phenotypes of these tagged strains compared to the wild-type (de Oliveira Bruder Nascimento et al., 2016; **Supplementary Figure S1**). Protein extracts were prepared from wild-type, SakA:GFP and SakA:GFP MpkC:3xHA strains that were untreated or treated with sorbitol 1.0 M or CR 300 μ g/ml for 10, 30 and 60 min. **Figure 2** shows that pull-down of SakA:GFP results in co-purification of MpkC only in the strain expressing SakA:GFP MpkC:3xHA and not in a wild-type or SakA:GFP strains. Moreover, this interaction occurs even in the absence of stress conditions (without Sorbitol and CR). We also tried to IP MpkC:3xHA and SakA:GFP first before probing for MpkC with an anti-HA or anti-GFP antibody, but the MpkC:3xHA and SakA:GFP tags interacted with the IP resin in control conditions, therefore de-validating the assay (data not shown).

SakA Associates With the MAPK MpkA and the Protein Phosphatase PtcB

As both MpkA and PtcB have been implicated in the HOG and CWI pathway and here they were identified in the SakA interactome in presence of CR (**Figure 3A** and **Tables 2, 4**), we used pull-down experiments to validate these interactions. We introduced into SakA:GFP strain, a plasmid expressing MpkA or PtcB tagged at C-terminus with HA tag. We showed that HA-tagging did not affect the phenotypes of these strains compared to the wild-type (**Supplementary Figures S2, S3**). We carried out Co-IP assays using GFP-Trap beads with the wild-type, SakA:GFP, SakA:GFP MpkA:3xHA (**Figure 3B**) and SakA:GFP PtcB:3xHA (**Figure 3C**) that were untreated or treated with CR 300 μ g/ml (10, 30, and 60 min). Interaction of HA tagged MpkA with PtcB were then identified with HA antibody via Western blot. The results of the immunoblotting analysis showed that MpkA and PtcB were only co-immunoprecipitated from the SakA:GFP MpkA:3xHA (**Figure 3B**) and SakA:GFP PtcB:3xHA (**Figure 3C**), respectively, illustrating that these two protein associate with SakA. Moreover, our results show that SakA-PtcB interaction occurs only in presence of CR stress (**Figure 3C**). We also tried to IP MpkA:3xHA or PtcB:3xHA and SakA:GFP first before probing for MpkA or PtcB with an anti-HA or anti-GFP antibody, but once more the MpkA:3xHA or PtcB:3xHA and SakA:GFP tags interacted with the IP resin

²<https://bio.tools/fungifun>

TABLE 1 | Selected proteins interacting with SakA::GFP identified during osmotic stress.

Accession	Description	Time (min) 10	Time (min) 30	Time (min) 60	MW (kDa)	calc. pl
Signal transduction						
Afu1g12940	sakA putative mitogen-activated protein kinase (MAPK) with predicted roles in the osmotic and oxidative stress responses; involved in sensing nitrogen in the medium	I*	I	I	41.9	5.60
Afu4g13170	cpcB G-protein complex beta subunit; conidia-enriched protein; immunoreactive	NI**	NI	I	35.0	6.52
Afu6g02230	glkA putative glucokinase; conidia-enriched protein; transcripts extremely abundant in resting conidia. Levels decline during germination; transcript induced by exposure to human airway epithelial cells	NI	NI	I	54.4	5.50
Afu2g03490	Putative calcium/calmodulin-dependent protein kinase; calcium induced; transcript induced by exposure to human airway epithelial cells	I	NI	I	72.1	6.87
Afu6g06720	Putative protein serine/threonine kinase	I	NI	I	99.8	9.64
Afu6g12170	fkbp1 putative FK506-binding protein (FKBP)-type peptidyl-prolyl <i>cis-trans</i> isomerase; transcript induced by exposure to human airway epithelial cells	NI	NI	I	12.1	7.25
Afu1g08840	Ortholog(s) have guanylate kinase activity. Role in GMP metabolic process and cytoplasm. nucleus localization	NI	NI	I	25.5	7.43
Cell wall						
Afu3g12690	glfA putative UDP-galactopyranose mutase. enzyme in the first step of galactofuranose biosynthesis; mutant unmasks mannan residues on the cell surface. which is thought to contribute to increased cell adhesion	I	NI	I	59.4	6.93
Afu7g02180	Ortholog(s) have UDP-N-acetylglucosamine diphosphorylase activity. Role in UDP-N-acetylglucosamine biosynthetic process and cytosol. Nucleus localization	NI	NI	I	56.7	6.10
Transcription factors						
Afu3g09820	dvrA C2H2 zinc finger domain protein; putative ortholog of <i>C. albicans</i> Bcr1p; mutants are hypervirulent in neutropenic mice and in flies	NI	I	I	73.2	9.48
Afu3g11170	Ortholog(s) have sequence-specific DNA binding activity and role in carotenoid biosynthetic process, circadian rhythm, conidiophore development	NI	I	I	84.2	6.71
Afu3g13870	nsdD Putative GATA-type transcriptional activator; required during an early stage of mating; plays a role in resistance toward cell wall stress	I	NI	I	53.0	9.26
Osmolytes/trehalose						
Afu3g12100	Putative trehalase phosphorylase with a predicted role in the glucose-1-phosphate pathway	NI	NI	I	78.3	6.10
Afu2g04010	tpsB putative trehalose-6-phosphate synthase; role in trehalose biosynthesis; expression up during heat shock; hypervirulent in combination with tpsA mutation. In a mouse model of pulmonary infection; predicted gene pair with AFUA_6G12950	NI	I	NI	53.8	7.43
Afu4g11510	srb1 GDP-mannose pyrophosphorylase, which catalyzes the synthesis of GDP-mannose from GTP and mannose-1-phosphate in cell wall biosynthesis	NI	NI	I	40.1	7.49
Heat shock proteins/chaperones						
Afu2g02320	Hsp70 chaperone	NI	NI	I	61.7	5.15
Lipids						
Afu3g06550	Has domain(s) with predicted phosphatase activity. Phosphoserine phosphatase activity and role in L-serine biosynthetic process. metabolic process	I	I	I	51.8	5.90
Afu5g05500	Ortholog(s) have phosphoglycerate dehydrogenase activity. Role in serine family amino acid biosynthetic process and cytosol localization	NI	I	I	45.9	7.01
Afu3g10660	erg13 putative hydroxymethyl glutaryl-coenzyme A synthase with a predicted role in ergosterol biosynthesis; SrbA-regulated during hypoxia	NI	NI	I	50.8	6.55

*I, identified. **NI, non-identified.

in control conditions, therefore de-validating the assay (data not shown). All together, these data show that the interactions found in the SakA and MpkC interactomes could be robustly validated using Co-IP approach.

Molecular Characterization of PakA^{STE20}

Here we identified PakA, the homolog of the *S. cerevisiae* sexual developmental serine/threonine kinase (Afu2g04680)

Ste20, as a component of the SakA/MpkC MAPK pathway. To gain an insight into the function of the Ste20 homolog in *A. fumigatus*, a *pakA* null mutant, and complemented strains were constructed (Supplementary Figure S4). The wild-type, $\Delta pakA$ and $\Delta pakA::pakA^+$ strains were grown in minimal medium (MM) and exposed to agents that affect CWI pathway, including CR, calcofluor white (CFW), and the echinocandin caspofungin (Figures 4A–C). The $\Delta pakA$ strain showed radial

TABLE 2 | Selected proteins interacting with MpkC::GFP identified during osmotic stress.

Accession	Description	Time (min) 10	Time (min) 30	Time (min) 60	MW (kDa)	calc. pI
Signal transduction						
Afu1g04950	glcA ortholog(s) have protein serine/threonine phosphatase activity	NI**	I*	NI	28.3	4.72
Afu2g16520	pld2 putative phospholipase D; calcium induced; repressed by exposure to artemisinin	NI	NI	I	64.7	7.65
Afu4g13720	mpkA mitogen-activated protein kinase; activated by phosphorylation; role in cell wall signaling and the oxidative stress response; mpkA(p)-lacZ expression increased by cell wall disturbing compounds; required for adaptation to iron starvation	NI	I	NI	48.4	5.88
Afu5g09100	mpkC putative mitogen activated protein kinase (MAPK); involved in the oxidative stress response; transcript abundance increases in response to carbon source and oxidative stress	I	I	I	43.3	5.76
Afu6g02230	glkA putative glucokinase; conidia-enriched protein; transcripts extremely abundant in resting conidia. Levels decline during germination; transcript induced by exposure to human airway epithelial cells	NI	NI	I	54.4	5.50
Afu2g05910	hxA putative hexokinase; protein induced by heat shock	NI	NI	I	68.2	6.14
Afu6g08580	fkbp4 putative FK506-binding protein (FKBP)-type peptidyl-prolyl <i>cis</i> -trans isomerase	NI	I	I	53.0	4.55
Afu6g12170	fkbp1 putative FK506-binding protein (FKBP)-type peptidyl-prolyl <i>cis</i> -trans isomerase; transcript induced by exposure to human airway epithelial cells	NI	NI	I	12.1	7.25
Afu4g13170	cpcB G-protein complex beta subunit; conidia-enriched protein; immunoreactive	NI	NI	I	35.0	6.52
Afu1g12940	sakA putative mitogen-activated protein kinase (MAPK) with predicted roles in the osmotic and oxidative stress responses; involved in sensing nitrogen in the medium	I	NI	I	21.0	5.71
Afu1g08840	Ortholog(s) have guanylate kinase activity. Role in GMP metabolic process and cytoplasm. Nucleus localization	NI	NI	I	61.0	6.34
Cell wall						
Afu7g02180	Ortholog(s) have UDP-N-acetylglucosamine diphosphorylase activity. Role in UDP-N-acetylglucosamine biosynthetic process and cytosol. Nucleus localization	NI	NI	I	56.7	6.10
Afu3g12690	glfA putative UDP-galactopyranose mutase. Enzyme in the first step of galactofuranose biosynthesis; mutant unmasks mannan residues on the cell surface, which is thought to contribute to increased cell adhesion	NI	NI	I	59.4	6.93
Afu6g12400	fkx1 putative 1,3-beta-glucan synthase catalytic subunit. Major subunit of glucan synthase; predicted transmembrane protein; and essential	NI	I	NI	218.0	8.15
Transcription factors						
Afu3g11170	Ortholog(s) have sequence-specific DNA binding activity and role in carotenoid biosynthetic process, circadian rhythm, and conidiophore development	NI	I	I	84.2	6.71
Osmolytes/trehalose						
Afu3g12100	Putative trehalase phosphorylase with a predicted role in the glucose-1-phosphate pathway	NI	NI	I	78.3	6.10
Afu2g10660	mpdA putative mannitol-1-phosphate dehydrogenase; conidia-enriched protein; protein induced by heat shock; reacts with rabbit immunosera exposed to conidia; no human homolog	NI	NI	I	27.5	5.03
Afu4g11510	srb1 GDP-mannose pyrophosphorylase. which catalyzes the synthesis of GDP-mannose from GTP and mannose-1-phosphate in cell wall biosynthesis	NI	NI	I	40.1	7.49
Afu3g11640	Homoserine dehydrogenase; no human homolog	I	NI	NI	38.5	5.87
Afu2g03810	hosA putative histone deacetylase	NI	I	I	28.3	7.87
Heat shock proteins/chaperones						
Afu2g02320	Hsp70 chaperone	NI	NI	I	41.3	5.99
Afu7g01860	sti1 putative heat shock protein; protein levels increase in response to amphotericin B; transcript induced by exposure to human airway epithelial cells; induced by gliotoxin exposure	NI	NI	I	64.3	5.58
Afu1g06710	Ortholog(s) have chaperonin-containing T-complex. Nucleus localization	NI	NI	I	210.7	6.05
Afu1g01740	Ortholog(s) have unfolded protein binding activity. Role in protein folding and chaperonin-containing T-complex. Nucleus localization	NI	NI	I	51.8	5.90
Afu6g07540	Ortholog(s) have chaperonin-containing T-complex localization	NI	NI	I	59.8	5.52

(Continued)

TABLE 3 | Selected proteins interacting with SakA::GFP identified during cell wall stress.

Accession	Description	Time (min) 5	Time (min) 15	Time (min) 30	Time (min) 60	MW (kDa)	calc. pI
Signal transduction							
Afu1g04950	GlcA Ortholog(s) have protein serine/threonine phosphatase activity	NI**	I*	NI	NI	37.1	5.78
Afu1g09280	PtcB putative type 2C protein phosphatase (PP2C) involved in dephosphorylation of SakA MAP kinase in response to osmotic stress	NI	I	I	NI	46.6	4.79
Afu1g12940	SakA putative mitogen-activated protein kinase (MAPK) with predicted roles in the osmotic and oxidative stress responses; involved in sensing nitrogen in the medium	I	I	I	I	41.9	5.60
Afu2g03490	Putative calcium/calmodulin-dependent protein kinase; calcium induced; transcript induced by exposure to human airway epithelial cells	I	I	I	I	72.1	6.87
Afu2g04680	PakA protein serine/threonine kinase; similar to Ste20. a sexual development regulator	I	I	I	I	89.6	9.44
Afu2g10620	Ortholog(s) have protein serine/threonine kinase activity YPK1	NI	I	NI	NI	70.9	7.65
Afu3g07680	Putative ran GTPase activating protein	NI	I	I	NI	46.2	4.68
Afu3g10000	cAMP-dependent protein kinase regulatory subunit; induced by endothelial cells and human airway epithelial cells; mutants sensitive to storage in water and contain more nuclei than wild-type; up-regulated in neutrophil-exposed conidia	NI	NI	I	NI	44.5	5.19
Afu3g12530	PkhB putative sensor histidine kinase/response regulator; transcript induced by exposure to human airway epithelial cells	NI	I	NI	I	213.4	6.52
Afu4g10050	Calmodulin			I		17.0	4.27
Afu6g02230	GlcA putative glucokinase; conidia-enriched protein; transcripts extremely abundant in resting conidia. levels decline during germination; transcript induced by exposure to human airway epithelial cells	NI	NI	I	NI	54.4	5.50
Afu8g04810	Ortholog(s) have protein serine/threonine kinase activity cka1. Alpha' catalytic subunit of casein kinase 2 (CK2)	NI	I	I	NI	39.5	7.31
Afu3g10300	Putative galactokinase with a role in galactose catabolism; transcript up-regulated in conidia exposed to neutrophils	NI	I	I	NI	57.6	6.65
Afu1g05610	Putative protein phosphatase 2a 65kd regulatory subunit; conidia-enriched protein; protein induced by heat shock	NI	NI	I	NI	69.1	4.86
Cell wall							
Afu7g02180	Ortholog(s) have UDP-N-acetylglucosamine diphosphorylase activity. Role in UDP-N-acetylglucosamine biosynthetic process and cytosol. Nucleus localization	NI	NI	I	NI	56.7	6.10
Afu1g06210	agm1 N-acetylphosphoglucosamine mutase. Involved in chitin biosynthesis; essential	NI	I	NI	NI	61.5	6.11
Afu2g05340	gel4 essential 1,3-beta-glucanase/transferase. GPI-anchored to the plasma membrane; constitutively expressed during hyphal growth; and hypoxia induced protein	NI	I	NI	NI	57.1	5.01
Transcription factors							
Afu3g13870	NsdD putative GATA-type transcriptional activator; required during an early stage of mating; plays a role in resistance toward cell wall stress	I	I	NI	NI	53.0	9.26
Afu6g10470	Zpr1 putative zinc finger protein; transcript highly induced during conidial germination	NI	I	I	NI	53.5	4.67
Osmolytes/trehalose							
Afu1g09930	Gcy1 putative glycerol dehydrogenase; protein level decreases upon heat shock; transcript up-regulated in conidia exposed to neutrophils	NI	I	I	NI	39.1	8.84
Afu6g07620	GDP-mannose pyrophosphorylase A; repressed by exposure to artemisinin	NI	NI	I	NI	48.3	7.30
Afu3g12100	Putative trehalase phosphorylase with a predicted role in the glucose-1-phosphate pathway	NI	I	I	NI	78.3	6.10
Afu2g04010	TpsB putative trehalose-6-phosphate synthase; role in trehalose biosynthesis; expression up during heat shock; hypervirulent in combination with tpsA mutation. In a mouse model of pulmonary infection; predicted gene pair with AFUA_6G12950	I	NI	NI	I	53.8	7.43
Afu2g10660	MpdA putative mannitol-1-phosphate dehydrogenase; conidia-enriched protein; protein induced by heat shock; reacts with rabbit immunosera exposed to conidia; and no human homolog	NI	I	NI	NI	43.0	5.90
Afu1g02150	Ortholog(s) have glycerol-3-phosphate dehydrogenase [NAD+] activity and role in NADH oxidation. Cell-abiotic substrate adhesion. glycerol biosynthetic process. Intracellular accumulation of glycerol	NI	I	I	NI	56.4	8.19

(Continued)

TABLE 2 | Continued

Accession	Description	Time (min) 10	Time (min) 30	Time (min) 60	MW (kDa)	calc. pl
Lipids						
Afu5g05500	Ortholog(s) have phosphoglycerate dehydrogenase activity. Role in serine family amino acid biosynthetic process and cytosol localization	NI	I	I	45.9	7.01
Afu2g01010	Putative myo-inositol-phosphate synthase; transcript up-regulated in conidia exposed to neutrophils	NI	NI	I	73.9	9.86
Afu3g06550	Has domain(s) with predicted phosphatase activity. Phosphoserine phosphatase activity and role in L-serine biosynthetic process. Metabolic process	I	I	NI	64.3	7.06
Afu6g14200	erg10 putative acetyl-CoA acetyltransferase; transcript up-regulated in conidia exposed to neutrophils; SrbA-regulated during hypoxia	NI	NI	I	45.7	8.95
Afu2g09910	f4 putative long chain fatty acid CoA ligase; fatty acid activator; hypoxia repressed protein	I	NI	NI	163.9	9.36

*I, identified. **NI, non-identified.

growth similar to the wild-type strain in MM (Figure 4). The $\Delta pakA$ strain was slightly more sensitive to CR (Figure 4A) and showed similar sensitivity to high sorbitol concentrations when compared to the wild-type strain (data not shown). The $\Delta pakA$ was sensitive to CFW and caspofungin stresses (Figures 4B,C), and the loss of the caspofungin paradoxical effect (CPE, a phenomenon where high caspofungin concentrations revert the anticipated inhibition of *A. fumigatus* growth; Steinbach et al., 2015; Figure 4C).

Finally, to further characterize the possible effect of PakA on pathogenicity of *A. fumigatus*, an *Galleria mellonella* model which had been demonstrated as a good model to evaluate fungal pathogenicity was used (Slater et al., 2011). In the *G. mellonella* model, infection by either of wild-type or the complementing strains resulted in 100% mortality 8 and 7 days post-infection, respectively (Figure 5). However, the $\Delta pakA$ mutant strain showed 100 % mortality 10 days post-infection, which was statistically different to the wild-type, and complementing strains according to the Mantel-Cox and Gehan-Brestow-Wilcoxon tests (p -values 0.0041 and 0.0124, respectively). These results suggest that the lack of *pakA* attenuated the *A. fumigatus* virulence in this animal model.

DISCUSSION

Fungi live in diverse environments ranging from soil to mammalian host. Fungi are also exposed to many stressing conditions including heat shock, oxidative stress, osmotic and pH changes, chemical challenges, and nutrient limitations. These stress conditions can be found either in natural habitats or in animal and human hosts during the infection process (Brown and Goldman, 2016). Exposure of fungal cells to these stress conditions leads to the activation of some MAPK cascades (Pearson et al., 2001; Rispaill et al., 2009). In fungi the signaling through the MAPK cascades result in altered gene expression that regulates many processes including pheromone response, filamentous growth, biosynthesis of cell wall components, the HOG pathway, the establishment of virulence, and mediation of drug resistance (Bahn et al., 2005; Monge et al., 2006;

Román et al., 2007; Valiante et al., 2008, 2009, 2015a,b; de Castro et al., 2014; Altwasser et al., 2015; Bom et al., 2015; Winkelströter et al., 2015a,b).

In *A. fumigatus*, Hog1 orthologs SakA and MpkC show multifunctional roles. In addition to the role played in the osmotic stress response, these MAPKs also regulate stress response to stimuli such as oxidative stress, cell wall damaging agents (de Oliveira Bruder Nascimento et al., 2016) and also play a role in caspofungin adaptation, carbon source utilization, and collaborate during virulence establishment (Reyes et al., 2006; Altwasser et al., 2015; Valiante et al., 2015a; de Oliveira Bruder Nascimento et al., 2016). *A. nidulans* SakA and MpkC not only physically interact, but also show opposite and common functions during stress responses and development (Jaimes-Arroyo et al., 2015; Garrido-Bazán et al., 2018). However, little is known about the mechanisms by which SakA and MpkC execute their signaling functions.

We have identified by mass spectrometry many common SakA and MpkC protein interactors, suggesting they perform similar roles. Pull-down experiments strongly indicated SakA and MpkC are physically interacting, possibly forming a protein complex which regulates these down stream processes under specific conditions. Subsequently, by using SakA GFP-tag pull-down and mass spectrometry analysis, we showed that SakA interacts not only with MpkC, but also with the CWI pathway MAPK MpkA, the HOG response phosphatase PtcB and other proteins involved in signal transduction pathways, biosynthesis of the cell wall, transcription factors, heat shock proteins, and chaperones. Previously, we proposed that SakA and MpkC are interacting and that MpkC could be a modulator of SakA during HOG and CWI pathways, since SakA appears to play a major role in response to several types of stresses (de Oliveira Bruder Nascimento et al., 2016). In this work we proved this interaction by using Co-IP experiments and MpkC was showed to be continuously associated with SakA (stress and no stress). This resembles what happens in *A. nidulans* where MpkC was found to be linked with SakA with and without oxidative stress (Jaimes-Arroyo et al., 2015). Furthermore, we showed previously that both MpkC:GFP and SakA:GFP translocated to the nucleus upon osmotic stress, with SakA:GFP showing

TABLE 3 | Selected proteins interacting with SakA::GFP identified during cell wall stress.

Accession	Description	Time (min) 5	Time (min) 15	Time (min) 30	Time (min) 60	MW (kDa)	calc. pl
Signal transduction							
Afu1g04950	GlcA Ortholog(s) have protein serine/threonine phosphatase activity	NI**	I*	NI	NI	37.1	5.78
Afu1g09280	PtcB putative type 2C protein phosphatase (PP2C) involved in dephosphorylation of SakA MAP kinase in response to osmotic stress	NI	I	I	NI	46.6	4.79
Afu1g12940	SakA putative mitogen-activated protein kinase (MAPK) with predicted roles in the osmotic and oxidative stress responses; involved in sensing nitrogen in the medium	I	I	I	I	41.9	5.60
Afu2g03490	Putative calcium/calmodulin-dependent protein kinase; calcium induced; transcript induced by exposure to human airway epithelial cells	I	I	I	I	72.1	6.87
Afu2g04680	PakA protein serine/threonine kinase; similar to Ste20. a sexual development regulator	I	I	I	I	89.6	9.44
Afu2g10620	Ortholog(s) have protein serine/threonine kinase activity YPK1	NI	I	NI	NI	70.9	7.65
Afu3g07680	Putative ran GTPase activating protein	NI	I	I	NI	46.2	4.68
Afu3g10000	cAMP-dependent protein kinase regulatory subunit; induced by endothelial cells and human airway epithelial cells; mutants sensitive to storage in water and contain more nuclei than wild-type; up-regulated in neutrophil-exposed conidia	NI	NI	I	NI	44.5	5.19
Afu3g12530	PkhB putative sensor histidine kinase/response regulator; transcript induced by exposure to human airway epithelial cells	NI	I	NI	I	213.4	6.52
Afu4g10050	Calmodulin			I		17.0	4.27
Afu6g02230	GlcA putative glucokinase; conidia-enriched protein; transcripts extremely abundant in resting conidia. levels decline during germination; transcript induced by exposure to human airway epithelial cells	NI	NI	I	NI	54.4	5.50
Afu8g04810	Ortholog(s) have protein serine/threonine kinase activity cka1. Alpha' catalytic subunit of casein kinase 2 (CK2)	NI	I	I	NI	39.5	7.31
Afu3g10300	Putative galactokinase with a role in galactose catabolism; transcript up-regulated in conidia exposed to neutrophils	NI	I	I	NI	57.6	6.65
Afu1g05610	Putative protein phosphatase 2a 65kd regulatory subunit; conidia-enriched protein; protein induced by heat shock	NI	NI	I	NI	69.1	4.86
Cell wall							
Afu7g02180	Ortholog(s) have UDP-N-acetylglucosamine diphosphorylase activity. Role in UDP-N-acetylglucosamine biosynthetic process and cytosol. Nucleus localization	NI	NI	I	NI	56.7	6.10
Afu1g06210	agm1 N-acetylphosphoglucosamine mutase. Involved in chitin biosynthesis; essential	NI	I	NI	NI	61.5	6.11
Afu2g05340	gel4 essential 1.3-beta-glucanotransferase. GPI-anchored to the plasma membrane; constitutively expressed during hyphal growth; and hypoxia induced protein	NI	I	NI	NI	57.1	5.01
Transcription factors							
Afu3g13870	NsdD putative GATA-type transcriptional activator; required during an early stage of mating; plays a role in resistance toward cell wall stress	I	I	NI	NI	53.0	9.26
Afu6g10470	Zpr1 putative zinc finger protein; transcript highly induced during conidial germination	NI	I	I	NI	53.5	4.67
Osmolytes/trehalose							
Afu1g09930	Gcy1 putative glycerol dehydrogenase; protein level decreases upon heat shock; transcript up-regulated in conidia exposed to neutrophils	NI	I	I	NI	39.1	8.84
Afu6g07620	GDP-mannose pyrophosphorylase A; repressed by exposure to artemisinin	NI	NI	I	NI	48.3	7.30
Afu3g12100	Putative trehalase phosphorylase with a predicted role in the glucose-1-phosphate pathway	NI	I	I	NI	78.3	6.10
Afu2g04010	TpsB putative trehalose-6-phosphate synthase; role in trehalose biosynthesis; expression up during heat shock; hypervirulent in combination with tpsA mutation. In a mouse model of pulmonary infection; predicted gene pair with AFUA_6G12950	I	NI	NI	I	53.8	7.43
Afu2g10660	MpdA putative mannitol-1-phosphate dehydrogenase; conidia-enriched protein; protein induced by heat shock; reacts with rabbit immunosera exposed to conidia; and no human homolog	NI	I	NI	NI	43.0	5.90
Afu1g02150	Ortholog(s) have glycerol-3-phosphate dehydrogenase [NAD+] activity and role in NADH oxidation. Cell-abiotic substrate adhesion. glycerol biosynthetic process. Intracellular accumulation of glycerol	NI	I	I	NI	56.4	8.19

(Continued)

TABLE 3 | Continued

Accession	Description	Time (min) 5	Time (min) 15	Time (min) 30	Time (min) 60	MW (kDa)	calc. pI
Heat shock proteins/ chaperones							
Afu2g16020	Putative heat shock protein; transcript induced during conidial germination	NI	NI	I	NI	61.7	6.87
Afu1g01740	Ortholog(s) have unfolded protein binding activity. Role in protein folding and chaperonin-containing T-complex. Nucleus localization	NI	NI	I	NI	57.2	6.21
Afu6g07540	Ortholog(s) have chaperonin-containing T-complex localization	NI	I	I	NI	59.8	5.52
Afu3g14540	Hsp30 putative 30-kilodalton heat shock protein; conidia-enriched protein; protein levels increase in response to amphotericin B and hydrogen peroxide	NI	I	I	NI	20.5	6.55
Afu4g10010	Ortholog(s) have heat shock protein binding activity. Role in protein folding. Regulation of mitotic cell cycle and cytosol. Nucleus localization	NI	I	I	NI	54.5	5.00
Afu5g13920	wos2 putative Hsp90 binding co-chaperone; reacts with rabbit immunosera exposed to <i>A. fumigatus</i> conidia	NI	NI	I	NI	22.2	4.48
Afu7g01860	sti1 putative heat shock protein; protein levels increase in response to amphotericin B; transcript induced by exposure to human airway epithelial cells; induced by gliotoxin exposure	NI	I	I	NI	64.3	5.58
Afu2g02320	Hsp70 chaperone	NI	NI	I	NI	61.7	5.15
Pentose phosphate pathway oxidative branch							
Afu1g02980	Putative 6-phosphogluconolactonase; calcium downregulated	NI	I	I	NI	28.8	6.28
Afu3g08470	Glucose-6-phosphate 1-dehydrogenase	NI	NI	I	NI	58.9	7.01
Lipids							
Afu2g11340	Ortholog(s) have role in intracellular sterol transport and fungal-type vacuole lumen localization	NI	I	I	NI	27.3	5.94
Afu3g06550	Has domain(s) with predicted phosphatase activity. Phosphoserine phosphatase activity and role in L-serine biosynthetic process. Metabolic process	I	I	I	I	51.8	5.90
Afu5g05500	Ortholog(s) have phosphoglycerate dehydrogenase activity. Role in serine family amino acid biosynthetic process and cytosol localization	I	I	I	NI	45.9	7.01
Afu5g02450	erg20 putative polyprenyl synthetase; farnesyl-pyrophosphate synthetase; SrbA-regulated during hypoxia	NI	NI	I	NI	39.7	5.50
Afu5g05820	Ortholog(s) have homoserine kinase activity. Role in homoserine metabolic process. Threonine biosynthetic process and cytoplasm localization	NI	I	NI	NI	38.5	6.74
Afu5g03690	Ortholog(s) have phosphatidylinositol transporter activity	NI	I	I	I	46.1	4.84
Afu6g04970	Ortholog(s) have O-phospho-L-serine:2-oxoglutarate aminotransferase activity. Role in L-serine biosynthetic process. Purine nucleobase biosynthetic process and cytosol localization	NI	I	I	I	46.9	6.81
Afu3g11640	Homoserine dehydrogenase; no human homolog	I	I	I	I	38.5	5.87
Afu7g03740	cyp51B putative 14- α demethylase with a predicted role in ergosterol biosynthesis	NI	NI	I	NI	58.9	7.75
Afu4g06890	Cyp51A 14- α sterol demethylase; commonly mutated in drug resistant isolates	NI	I	NI	NI	58.0	8.53
Afu4g07130	mvd1 diphosphomevalonate decarboxylase; SrbA-regulated during hypoxia	NI	NI	I	NI	43.1	6.01
Afu4g03630	erg6 putative sterol 24-C-methyltransferase with a predicted role in ergosterol biosynthesis	NI	I	I	NI	42.5	6.43
Afu3g10660	erg13 putative hydroxymethyl glutaryl-coenzyme A synthase with a predicted role in ergosterol biosynthesis; SrbA-regulated during hypoxia	NI	I	I	NI	50.8	6.55
Afu3g09910	Ortholog(s) have phosphatidylcholine transporter activity and role in Golgi to plasma membrane transport, ascospore formation, mitotic cytokinesis, phospholipid transport, and regulation of filamentous growth	NI	I	I	NI	37.3	5.52
Afu3g08980	Ortholog(s) have threonine synthase activity. Role in threonine biosynthetic process and cytosol. Nucleus localization	NI	I	I	NI	59.2	6.20
Afu2g01010	Putative myo-inositol-phosphate synthase; transcript up-regulated in conidia exposed to neutrophils	NI	NI	I	NI	58.6	6.23

*I, identified. **NI, non-identified.

a quicker response (de Oliveira Bruder Nascimento et al., 2016). This data can explain the absence of MpkC co-purified with SakA after 10 min of sorbitol exposure. Our pull-down experiments

not only showed SakA-MpkC interaction, but also identified a common group of 78 and 256 proteins that potentially interact with both SakA and MpkC upon osmotic stress and

TABLE 4 | Selected proteins interacting with MpkC::GFP identified during cell wall stress.

Protein identifier	Description	Time (min) 5	Time (min) 15	Time (min) 30	Time (min) 60	MW (9 kDa)	calc. pI
Signal transduction							
Afu1g05610	Protein phosphatase 2a 65kd regulatory subunit; conidia-enriched protein; protein induced by heat shock	I*	NI**	NI	NI	69.1	4.86
Afu1g12940	sakA putative mitogen-activated protein kinase (MAPK) with predicted roles in the osmotic and oxidative stress responses; involved in sensing nitrogen in the medium	I	I	I	I	41.9	5.60
Afu2g13680	Putative calcium/calmodulin-dependent protein kinase; transcript induced by exposure to human airway epithelial cells	NI	NI	I	NI	48.7	6.49
Afu4g10050	Calmodulin			I		17.0	4.27
Afu5g09100	MpkC mitogen activated protein kinase (MAPK)	I	I	I	I	43.3	5.76
	mpkB putative mitogen-activated protein kinase (MAPK)	I	NI	NI	NI	40.8	6.90
Afu4g13720	MpkA mitogen-activated protein kinase	NI	I	NI	I	48.4	5.88
Afu6g02230	GikA putative glucokinase	NI	NI	I	NI	54.4	5.50
Afu5g02560	Ortholog(s) have protein serine/threonine phosphatase activity	NI	NI	NI	I	73.8	6.99
Afu3g10300	Putative galactokinase with a role in galactose catabolism	I	NI	I	NI	57.6	6.65
Transcription factors							
Afu3g13870	nsdD putative GATA-type transcriptional activator; required during an early stage of mating; plays a role in resistance toward cell wall stress	NI	I	NI	NI	53.0	9.26
Afu6g10470	Zpr1 putative zinc finger protein; transcript highly induced during conidial germination	NI	I	NI	NI	53.5	4.67
Afu3g11170	Ortholog(s) have sequence-specific DNA binding activity and role in carotenoid biosynthetic process, circadian rhythm, and conidiophore development	NI	I	I	NI	84.2	6.71
Cell wall							
Afu7g02180	Ortholog(s) have UDP-N-acetylglucosamine diphosphorylase activity. Role in UDP-N-acetylglucosamine biosynthetic process and cytosol. Nucleus localization	NI	NI	I	NI	56.7	6.10
Afu7g05450	sun1 novel beta-1,3-glucan modifying enzyme involved in fungal morphogenesis	NI	NI	I	NI	43.5	5.48
Afu3g14420	chsG putative class III chitin synthase	NI	NI	NI	I	101.6	8.15
Osmolytes/trehalose							
Afu4g11510	srb1 GDP-mannose pyrophosphorylase, which catalyzes the synthesis of GDP-mannose from GTP and mannose-1-phosphate in cell wall biosynthesis	NI	NI	I	NI	40.1	7.49
Afu1g09840	gcy1 putative glycerol dehydrogenase; protein level decreases upon heat shock; transcript up-regulated in conidia exposed to neutrophils	NI	NI	NI	I	39.1	8.84
Afu3g12100	Putative trehalase phosphorylase with a predicted role in the glucose-1-phosphate pathway	NI	I	I	NI	78.3	6.10
Afu2g04010	tpsB putative trehalose-6-phosphate synthase; role in trehalose biosynthesis; expression up during heat shock; hypervirulent in combination with tpsA mutation. In a mouse model of pulmonary infection	NI	I	NI	NI	53.8	7.43
Afu6g07620	GDP-mannose pyrophosphorylase A; repressed by exposure to artemisinin	NI	NI	I	NI	48.3	7.30
Afu2g04010	tpsB putative trehalose-6-phosphate synthase; role in trehalose biosynthesis; expression up during heat shock	NI	I	NI	NI	53.8	7.43
Heat shock proteins/chaperones							
Afu2g02320	Hsp70 chaperone	NI	NI	I	NI	61.7	5.15
Afu7g01860	sti1 putative heat shock protein; protein levels increase in response to amphotericin B; transcript induced by exposure to human airway epithelial cells; induced by gliotoxin exposure	NI	I	I	NI	64.3	5.58
Afu3g14540	Hsp30 putative 30-kilodalton heat shock protein; conidia-enriched protein; protein levels increase in response to amphotericin B and hydrogen peroxide	I	I	NI	NI	20.5	6.55
Afu5g13920	Wos2 Putative Hsp90 binding co-chaperone; reacts with rabbit immunosera exposed to <i>A. fumigatus</i> conidia	NI	NI	I	NI	22.2	4.48
Afu1g02980	Putative 6-phosphogluconolactonase; calcium downregulated	NI	I	NI	NI	2.8	6.28
Afu3g08470	Glucose-6-phosphate 1-dehydrogenase	NI	NI	I	NI	58.9	7.01

*I, identified. **NI, non-identified.

cell wall damage, respectively. It is clear that these common interactions confirm that SakA-MpkC interact with similar proteins in *A. fumigatus*.

Previous work showed that the phosphorylation level of the MpkA protein during osmotic stress and cell wall damage is regulated by SakA and MpkC. The $\Delta sakA$ mutant showed

reduced MpkA phosphorylation and the double $\Delta mpkC \Delta sakA$ demonstrated no detectable MpkA phosphorylation (de Oliveira Bruder Nascimento et al., 2016). Furthermore, Altwasser et al. (2015) using systems biology approach, demonstrated the activation and cross talk between the *A. fumigatus* MpkA and SakA pathways during treatment with increased caspofungin doses. These authors have demonstrated that after exposure to caspofungin there is a higher level of SakA phosphorylation in the $\Delta mpkA$ mutant. Additionally, caspofungin influenced intracellular transport inducing a further osmotic stress; however this osmotic stress is reduced under high concentrations of caspofungin (Chen et al., 2011). The data presented here shows that MpkA and SakA are interactive under unstressed conditions and during the cell wall stress. In addition, our data show that SakA and MpkC associate with some proteins involved in the biosynthesis of the cell wall during the presence of osmotic stress and cell wall damage. Collectively, these presented data indicate that SakA and MpkC are allowing the signal integration and information exchange between HOG and CWI pathways.

Among the proteins identified in the Co-IP experiments that might be directly related to SakA function include the phosphatase PtcB. Previously we identified PtcB as a phosphatase related to the HOG pathway. We have shown that the $\Delta ptcB$ strain has both increased phosphorylation of SakA and MpkA, and regulates the expression of osmo-dependent genes (Winkelströter et al., 2015a). Our results provide the new insights into the mechanisms by which PtcB influences the HOG pathway in *A. fumigatus*.

Our results show that *A. fumigatus* mutant lacking the kinase PakA, homolog of the *S. cerevisiae* Ste20, is sensitive to agents that affect CWI pathway, and PakA does not mediate the sensitivity to osmotic stress. In yeast, Ste20p is involved in cellular responses to

nutritional limitation and mating pheromone, and is required to establish cell polarity (Cvrcková et al., 1995; Pringle et al., 1995; Simon et al., 1995; Richman et al., 1999). In addition, Ste20p is necessary for osmotic stress response via the Sho1p branch of the HOG pathway in *S. cerevisiae* (Raitt et al., 2000). Vaga et al. (2014) showed by phosphoproteomic analyses that yeast Ste20p is a key mediator of the Hog1 phosphorylation in response to high osmolarity and mating pathways. In this work, we reported the interaction between SakA and PakA. We then showed for the first time that PakA kinase is a component of the SakA/MpkC MAPK pathway in *A. fumigatus* which contributed to cell wall and caspofungin stress, plus virulence.

Together, our data support the hypothesis that SakA and MpkC are part of an osmotic and general signal pathways involved in regulation of the response to the cell wall damage, oxidative stress, drug resistance, and establishment of infection. The identification and characterization of novel protein interactors that are directly linked to SakA and MpkC function vital to antifungal resistance and virulence, represent potential new targets for the development of new antifungals combating IA.

MATERIALS AND METHODS

Strains, Media, and Growth Conditions

All strains used in this study are listed in **Supplementary Table S13** in the supplemental material, and the primers used are listed in **Supplementary Table S14**. The *A. fumigatus* parental strain were CEA17 (pyrG+) *akuBKU80* and CEA17 (pyrG-) *akuBKU80* (da Silva Ferreira et al., 2006). The MpkC:GFP and SakA:GFP strains were constructed by

FIGURE 3 | SakA:GFP associates with MpkA and PtcB. **(A)** Interaction network performed by using STRING (<https://string-db.org/>) showing the interaction among SakA, MpkC, MpkA, PtcB, and PakA. **(B)** Co-IP of SakA:GFP, MpkA:3xHA, SakA:GFP, and PtcB:3xHA. Affinity purification assays from GFP-tagged SakA strain in the background of 3xHA-tagged MpkA or PtcB were performed with GFP-Trap and anti-HA beads to verify interactions upon cell wall stress. CB, coomassie blue staining.

de Oliveira Bruder Nascimento et al. (2016). Media were of two basic types. A complete medium [YAG: 2% (w/v) glucose, 0.5% (w/v) yeast extract, 2% (w/v) agar, trace elements] with three variants: YUU (YAG supplemented with 1.2 g/l each of uracil and uridine) and liquid YG or YUU medium of the same composition but without agar. A modified minimal medium (MM: 1% (w/v) glucose, original high nitrate salts, trace elements, 2% (w/v) agar, pH 6.5) was also used. Trace elements, vitamins, and nitrate salts were described by Kafer (1977).

Plasmid Constructions

All *A. fumigatus* genes were isolated from the strain CEA17 D'Enfert et al. (1996). The cassette for *pakA* deletion was constructed by *in vivo* recombination in *S. cerevisiae* as previously described by Colot et al. (2006). Thus, approximately 2.0 kb from the 5'-UTR and 3'-UTR flanking region of the targeted ORF regions was selected for primer design. The primers 5F (*pakA* pRS426 5fw) and 3R (*pakA* pRS426 3rv) contained a

short homologous sequence to the MCS of the plasmid pRS426. Both the 5- and 3- UTR fragments were PCR amplified from *A. fumigatus* genomic DNA (gDNA). The *pyrG* gene placed within the cassette as a prototrophic marker was amplified from pCDA21 plasmid. The deletion cassette was generated by transforming each fragment along with the plasmid pRS426 cut with BamHI/EcoRI into the *S. cerevisiae* strain SC94721 using the lithium acetate method (Schiestl and Gietz, 1989). The DNA from the transformants was extracted by the method described by Goldman et al. (2003). The cassette was PCR amplified from these plasmids utilizing TaKaRa Ex Taq^{TM} DNA Polymerase (Clontech Takara Bio) and used for *A. fumigatus* transformation. Southern blot analysis demonstrated that the transformation cassette had integrated homologously at the targeted loci (Supplementary Figure S4). The single gene deletion of the *pakA* was complemented by co-transforming a DNA fragment (approximately 1 kb from each 5' and 3' flanking regions plus the ORF) together with the pHAT α (Herrera-Estrella et al., 1990)

and selecting for hygromycin resistance in MM plates with 250 mg/ml of hygromycin B. Southern blot was used to confirm the reconstituted strain (Supplementary Figure S4). For the generation of 3xHA fusion fragments $mpkC::3xHA::ptrA$, $mpkA::3xHA::ptrA$ and $ptcB::3xHA::ptrA$, a portion of DNA consisting of the gene ORF and 5' UTR region, along with a 1-Kb segment of DNA consisting of the 3' UTR flanking region were amplified with the primers pairs listed in supplemental table SX, from CEA17 gDNA. The 0.8 kb $3xHA::trpC$ fusion was amplified with primers OZG916/trpC REV $ptrA$ from the pOB430 plasmid and a $ptrA$ fragment amplified from the plasmid pPTR. The cassette was generated by transforming each fragment along with the plasmid pRS426 cut with BamHI/EcoRI into the *S. cerevisiae* strain. These cassettes were then transformed into the CEA17 strain and verification of 3xHA tagged strains were confirmed via PCR reaction (Supplementary Figures S1–S4).

Southern blot and PCR analyses were used to demonstrate that the cassettes had integrated homologously at the targeted *A. fumigatus* loci. Genomic DNA from *A. fumigatus* was extracted by grinding frozen mycelia in liquid nitrogen and

then gDNA was extracted as previously described (Malavazi and Goldman, 2012). Standard techniques for manipulation of DNA were carried out as described (Sambrook and Russell, 2001). For Southern blot analysis, restricted chromosomal DNA

FIGURE 5 | *Aspergillus fumigatus* PakA contributes to virulence in *G. mellonella*. Comparative analysis of wild-type and mutant strains in *G. mellonella* animal model. Larvae in groups of 10 per strain were infected with a 5 μl suspension of conidia at a dose of 1×10^6 /larva. PBS, phosphate buffer saline.

fragments were separated on 1% agarose gel and blotted onto Hybond N+ nylon membranes (GE Healthcare). Probes were labeled using [α -³²P]dCTP using the Random Primers DNA Labeling System (Life Technologies). Labeled membranes were exposed to X-ray films, which were scanned for image processing. Southern blot and PCR schemes are shown in **Supplementary Figures S1–S4**.

Phenotypic Assays

The phenotypes of the deletion mutant $\Delta pakA$ were evaluated by radial growth in MM and in presence of agents that affect CWI, including CR, calcofluor white (CFW) and the echinocandin caspofungin. The experiments were performed using 5 μ l of a 2×10^7 conidia for the wild-type and mutant strain and grown for 96 h at 37°C.

Protein Interaction Network Analysis

For the analysis of the interaction networks, proteins identified by proteomics were analyzed using STRING³ considering medium confidence parameter, and all possible interaction parameters allowed (such as co-expression, experimental evidence, and co-occurrence, etc). Resulting interaction networks were further processed using *ad hoc* Perl scripts and plots were generated using Gephi⁴.

GFP-Tag Protein Purification and Identification by LC-MS/MS

To precipitate GFP-tag-labeled SakA and MpkC, protein crude extracts were prepared from wild-type, SakA:GFP and MpkC:GFP cultures grown for 24 h and further exposed to sorbitol 1.0 M (10, 30, and 60 min) or CR 300 μ g/mL (5, 15, 30, and 60 min). Crude protein extracts from mycelia were obtained by extraction from ground mycelia with B250 buffer (250 mM NaCl, 100 mM Tris-HCl pH 7.5, 10% glycerol, 1 mM EDTA and 0.1% NP-40) supplemented with 1.5 ml/L 1 M DTT, 2 tables/100 mL complete-mini protease inhibitor cocktail EDTA-free (Roche), 3 ml/L 0.5 M Benzamidine, 10 ml/L phosphatase inhibitors 100 $\times$ (10 M NaF, 5 M Na Vanadate, 8 M β -glycerol phosphate), and 10 ml/L 100 mM PMSF. Total protein lysates were submitted to centrifuge at 13,000 rpm at 4°C for 10 min, the supernatant was collected into a new eppendorf. Magnetics GFP-trap beads were equilibrated with lysis buffer B250 (20 μ l of beads into 500 μ l lysis buffer B250; de Assis et al., 2018) during 10 min, after then were collected using magnet hack and incubated with total protein lysate at 4°C during 3 h. After incubation the magnetics GFP-trap beads were collected using magnetic hack and the supernatant was removed. The magnetic GFP-trap beads were washed two times using 500 μ l lysis buffer B250 without DTT and one additional wash step was done with addition of DTT. The magnetics GFP-trap beads were collected and supernatant was removed. The LC-MS/MS identification was performed as described previously (Johnk et al., 2016). Digested peptides were separated using reversed-phase liquid

chromatography with an RSLCnano Ultimate 3000 system (Thermo Fisher Scientific) followed by mass identification with an Orbitrap Velos Pro mass spectrometer (Thermo Fisher Scientific). Chromatographically separated peptides were on-line ionized by nano-electrospray (nESI) using the Nanospray Flex Ion Source (Thermo Fisher Scientific) at 2.4 kV and continuously transferred into the mass spectrometer. Full scans within m/z of 300–1850 were recorded by the Orbitrap-FT analyzer at a resolution of 30,000 (using m/z 445.120025 as lock mass) with parallel data-dependent top 10 MS2-fragmentation in the LTQ Velos Pro linear ion trap. LCMS method programming and data acquisition was performed with the software XCalibur 2.2 (Thermo Fisher Scientific) and method/raw data validation with the program RawMeat 2.1 (Vast Scientific). MS/MS data processing for protein analysis and identification was done with either MaxQuant quantitative proteomic software in conjunction with Perseus software for statistical analysis or the Proteome Discoverer 1.3 (PD, Thermo Fisher Scientific) and the Discoverer Daemon 1.3 (Thermo Fisher Scientific) software using the Sequest (and/or Mascot) peptide analysis algorithm(s) and organism-specific taxon-defined protein databases extended by the most common contaminants.

Co-IPs With GFP-Trap and Anti-HA Magnetic Beads

To perform co-IP assays, C-terminal HA-tagged MpkC, MpkA, and PtcB strains were generated in the SakA:GFP background. The strains were grown for 16 h in MM and further exposed to sorbitol 1.0 M (10, 30, and 60 min) or CR 300 μ g/mL (5, 15, 30, and 60 min). GFP-Trap co-IP experiments were performed as previously reported (Manfiolli et al., 2017). To perform reciprocal coimmunoprecipitation assays mycelia were frozen with liquid nitrogen, ground, and 500 mg was resuspended in 1 ml of B250 buffer (see above). Samples were centrifuged at 16,100 $\times g$ for 10 min at 4°C. Supernatant was collected, and a Bradford assay (BioRad) was carried out to measure protein content. The same amount of protein for each sample was added to 20 μ l of Dynabeads Protein A (Thermo Fisher Scientific) previously incubated with monoclonal anti-HA antibody (Sigma). The resin was washed three times with resuspension buffer prior to incubation. Cell extracts and resin were then incubated with shaking at 4°C for 2 h. After incubation, the resin was washed three times in resuspension buffer by placing the tube in a DynaMag^{TM} magnet. To release the proteins from the resin, samples were incubated with Sample Buffer and boiled at 98°C for 5 min. Proteins were transferred from a 10% SDS-PAGE gel onto a nitrocellulose membrane for a Western blot assay using a Trans-Blot turbo transfer system (Bio-Rad). GFP-tagged SakA was detected using a rabbit anti-GFP antibody (Abcam) at 1:2,000 dilution and a goat anti-rabbit I gG horseradish peroxidase (HRP) antibody (Cell Signaling Technology) at 1:10,000 dilution. For the HA-tagged proteins detection, a mouse monoclonal anti-HA antibody (Sigma) was used at 1:2,000 dilution as a primary antibody followed by an anti-mouse I gG HRP conjugate

³<https://string-db.org/>

⁴<https://gephi.org/>

(Cell Signaling Technology) used at 1:10,000 dilution as a secondary antibody.

Galleria mellonella Experiments

Galleria mellonella larvae were obtained by breeding adult moths (Fuchs et al., 2010). *G. mellonella* larvae of a similar size were selected (approximately 275–330 mg) and kept without food in glass container (Petri dishes), at 37°C, in darkness for 24 h prior to use. *A. fumigatus* conidia were obtained by growing on YAG media culture for 2 days. The conidia were harvested in PBS and filtered through a Miracloth (Calbiochem). The concentration of conidia was estimated by using hemocytometer, and resuspended at a concentration of 2.0×10^8 conidia/ml. The viability of the conidia was determined by incubating on YAG media culture, at 37°C, 48 h. Inoculum ($5 \mu\text{l}$ of a 2×10^8 conidia/ml) from the wild-type, mutant and complemented strains (1×10^6 conidia/larva) were used to investigate the virulence of *A. fumigatus* against *G. mellonella*. Ten *G. mellonella* in the final (sixth) instar larval stage of development were used per condition in all assays. The control group was the larvae inoculated with $5 \mu\text{l}$ of PBS to observe the killing due to physical trauma. The inoculum was performed by using Hamilton syringe (7000.5KH) and $5 \mu\text{l}$ into the haemocoel of each larva via the last left proleg. After, the larvae were incubated in glass container (Petri dishes) at 37°C in the dark. The larval killing was scored daily. Larvae were considered dead by presenting the absence of movement in response to touch.

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AUTHOR CONTRIBUTIONS

AM and NB wrote the manuscript. AM, EM, LA, LS, R-SR, and MU performed the experiments. NB and GG reviewed the manuscript. ÖB and GG designed the experiments and obtained the financial resources.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: <https://www.frontiersin.org/articles/10.3389/fmicb.2019.00918/full#supplementary-material>

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Mitogen activated protein kinases (MAPK) and protein phosphatases are involved in *Aspergillus fumigatus* adhesion and biofilm formation

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ABSTRACT

The main characteristic of biofilm formation is extracellular matrix (ECM) production. The cells within the biofilm are surrounded by ECM which provides structural integrity and protection. During an infection, this protection is mainly against cells of the immune system and antifungal drugs. *A. fumigatus* forms biofilms during static growth on a solid substratum and in chronic aspergillosis infections. It is important to understand how, and which, *A. fumigatus* signal transduction pathways are important for the adhesion and biofilm formation in a host during infection. Here we investigated the role of MAP kinases and protein phosphatases in biofilm formation. The loss of the MAP kinases MpkA, MpkC and SakA had an impact on the cell surface and the ECM during biofilm formation and reduced the adherence of *A. fumigatus* to polystyrene and fibronectin-coated plates. The phosphatase null mutants $\Delta sitA$ and $\Delta ptcB$, involved in regulation of MpkA and SakA phosphorylation, influenced cell wall carbohydrate exposure. Moreover, we characterized the *A. fumigatus* protein phosphatase PphA. The $\Delta pphA$ strain was more sensitive to cell wall-damaging agents, had increased β -(1,3)-glucan and reduced chitin, decreased conidia phagocytosis by *Dictyostelium discoideum* and reduced adhesion and biofilm formation. Finally, $\Delta pphA$ strain was avirulent in a murine model of invasive pulmonary aspergillosis and increased the released of tumor necrosis factor alpha (TNF- α) from bone marrow derived macrophages (BMDMs). These results show that MAP kinases and phosphatases play an important role in signaling pathways that regulate the composition of the cell wall, extracellular matrix production as well as adhesion and biofilm formation in *A. fumigatus*.

Introduction

Biofilms are composed by microorganisms attaching to each other, often associated with a surface, and typically encased within an extracellular matrix (ECM) complex polysaccharide. The ECM produces a structural scaffold for cohesion between cells, and to surfaces (Flemming & Wingender, 2010; O'Toole, 2003). In addition, the ECM can contribute to the retention of nutrients and water and also provide structural integrity and protection to the biofilm cells. In a human host this protection is mainly against cells of the immune system, and often has a role in resistance to antifungal therapies (Costerton et al., 1999; Donlan, 2001; Flemming & Wingender, 2010). The ECM of fungal biofilms is composed of different classes of macromolecules, such as proteins, carbohydrates, lipids and nucleic acids (Cegelski, 2015;

Martins et al., 2010; Reichhardt et al., 2015; Zarnowski et al., 2014).

The fungal pathogen *Candida albicans* frequently forms biofilms on implanted medical devices and the environmental circumstances strongly affect ECM production (Hawser et al., 1998). *C. albicans* biofilm development involves a series of sequential events. In the adherence step, the yeast cells adhere to a substrate. Then, the cells proliferate to establish microcolonies and produce elongated projections that yield the filamentous forms. In the maturation step occurs the accumulation of the ECM, biofilm expansion and increased drug resistance (Finkel & Mitchell, 2011). Initial investigations identified proteins, hexosamine, carbohydrates, phosphorus, uronic acid and extracellular DNA (eDNA) as components of the *C. albicans* ECM (Al-Fattani & Douglas, 2006; Baillie & Douglas, 2000). Several groups have since performed comprehensive analysis of the composition and

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relative abundance of each *C. albicans* ECM component. Unexpectedly, the β -1,3 glucan, an earlier identified sugar with important role in the ECM, was much less abundant when compared to β -1,6 glucan and α -1,6 mannan (Al-Fattani & Douglas, 2006; Faria-Oliveira et al., 2014; Thomas et al., 2006; Zarnowski et al., 2014). However, increased susceptibility to fluconazole treatments, both *in vivo* and *in vitro*, was observed when biofilms were treated with β -1,3 glucanase. Further studies have shown a role for β -1,3 glucan in the resistance to other antimicrobial agents in *C. tropicalis*, *C. parapsilosis*, and *C. glabrata* (Fernandes et al., 2015; Mitchell et al., 2013; Nett et al., 2010; Yi et al., 2011). Although there are similar polysaccharides found in the ECM and cell wall of *C. albicans*, several studies suggest that synthesis of the ECM polysaccharides is regulated by pathways distinct of those involved in cell wall synthesis (Chaffin, 2008). In *C. albicans*, ECM synthesis and biofilm production are regulated by specific transcription factors that govern morphological changes, adhesion and ECM production. The two main transcription factors are Bcr1p, a regulator that positively affect the expression of many adhesins during biofilm formation (Nobile & Mitchell, 2005; Srikantha et al., 2013), and Zap1p, which negatively regulates the expression of genes required for β -1,3 glucan production (Nobile et al., 2009). Fks1p, a β -1,3 glucan synthase, is involved directly in production of β -1,3 glucan and this enzyme controls the level of this ECM polysaccharide (Nett et al., 2010; Taff et al., 2012). The deletion of *SMI1* or *RLM1*, members of kinase C (PKC) pathway, resulted in lower levels of β -1,3 glucan in the ECM and in azole susceptibility (Nett et al., 2011). These phenotypes were repaired by *FKS1* overexpression in the deletion mutants. However, no effect was observed when upstream components of the PKC pathway were deleted (Nett et al., 2011). These results clearly demonstrate that the PKC pathway regulates in a distinct manner the mechanisms involved in biofilm ECM production compared to general cell wall integrity.

Aspergillus fumigatus, the principal airborne fungal pathogen, grows as a typical biofilm with hyphae surrounded by an ECM (Beauvais et al., 2007, 2014; Lousert et al., 2010). *A. fumigatus* biofilms can be produced during chronic pulmonary infections such as asthma, cystic fibrosis, obstructive pulmonary disease and allergic bronchopulmonary aspergillosis (Taylor et al., 2014; Xu et al., 2013). On rare occasions, *A. fumigatus* biofilms are found on medical devices for healthcare (Lousert et al., 2010; Ramage et al., 2011). The initial studies of ECM components of *A. fumigatus* biofilms showed the presence of galactomannan, galactosaminogalactan (GAG), α -1,3 glucan, proteins, polyols and melanin. Furthermore, eDNA has been detected in biofilms produced by *A. fumigatus*. Immunolabeling analysis of the *in vitro* secreted proteins in *A. fumigatus* ECM showed the presence of two major antigens, dipeptidylpeptidase V (DPPV) and catalase B (CatB), and one allergen, AspF1 (Rajendran et al., 2013). Several studies have demonstrated that the presence of GAG in the *A. fumigatus* ECM is an important virulence factor that permits the fungus to avoid the host innate immune system (Fontaine et al., 2011; Gravelat et al., 2013; Gresnigt et al., 2014; Robinet et al., 2014). Microarrays and RNA sequencing (RNAseq) analysis of *A. fumigatus* biofilms showed transcriptional up-regulation of hydrophobins and some important genes involved in biosynthesis of secondary metabolites. As expected, cell surface proteins and some proteins involved in adhesion, such as glycosylphosphatidylinositol (GPI)-anchored cell wall were found to be up-regulated in *A. fumigatus* biofilms (Gibbons et al., 2012; Muszkieta et al., 2013). It has been reported that *A. fumigatus* biofilm production increased resistance to azoles, echinocandins and polyenes (Beauvais et al., 2007; Mowat et al., 2007, 2008; Seidler et al., 2008). The mechanisms involved in the resistance of *A. fumigatus* biofilms to antifungal drugs include the increased expression of multidrug resistance (MDR) pumps (Muszkieta et al., 2013; Ramage et al., 2012), antioxidant defenses (Bruns et al., 2010; Delattin et al., 2014), Hsp90 (Robbins et al., 2011), and the presence of persister cells (Beauvais & Müller, 2009).

Despite these findings, the regulation of biofilm formation in *A. fumigatus* is relatively unknown. Some work showed that production of

GAG is dependent on proteins that regulate development, including StuA (Gravelat et al., 2013), MedA (Al Abdallah et al., 2012; Gravelat et al., 2010, 2013), SomA (Lin et al., 2015), and more recently PtaB, a lim-domain binding protein that regulates biofilm formation (Zhang et al., 2017). Recent studies by our group showed that SitA and PtcB phosphatases are important for ECM production and biofilm formation in *A. fumigatus* (Bom et al., 2015; Winkelströter et al., 2015a). Biofilms have the capacity to resist commonly used doses of antimicrobial agents, and this characteristic has become an important clinical issue. Thus, the characterization of proteins and pathways involved in biofilm production is essential to understand the genetic control of fungal biofilm development and the establishment of a biofilm in a host during *A. fumigatus* infection.

This work aimed to investigate the role of MAP kinases and protein phosphatases in biofilm formation. Here we presented evidence which support the role of *Aspergillus fumigatus* MAP kinases and phosphatases in controlling biofilm formation, ECM production, adhesion, cell wall composition as well as pathogenicity and virulence.

Material and methods

Ethics statement

The principles that guide our studies are based on the Declaration of Animal Rights ratified by the UNESCO in January 27, 1978 in its 8th and 14th articles. All protocols used in this study were approved by the local ethics committee for animal experiments from the Campus of Ribeirão Preto, Universidade de São Paulo (Permit Number: 08.1.1277.53.6; Studies on the interaction of *Aspergillus fumigatus* with animals). All animals were housed in groups of five within individually ventilated cages and were cared for in strict accordance with the principles outlined by the Brazilian College of Animal Experimentation (Princípios Éticos na Experimentação Animal – Colégio Brasileiro de Experimentação Animal, COBEA) and Guiding Principles for Research Involving Animals and Human Beings, American Physiological Society. All efforts were made to minimize suffering. Animals were clinically monitored at least twice daily and humanely sacrificed if moribund (defined by lethargy, dyspnoea, hypothermia and weight loss). All stressed animals were sacrificed by cervical dislocation.

Strains and media

The *A. fumigatus* strains used in this study are described in Table 1. Media were of two basic types. A complete medium with three variants: (2% (w/v) glucose, 0.5% (w/v) yeast extract, 2% (w/v) agar, trace elements), YUO (YAG supplemented with 1.2 g/L each of uracil and

Table 1
Strains used in this work.

Strain	Genotype	Source
Afs35	Wild-type strain	FGSC A1159
CEA17	Wild-type strain	Our laboratory
Ku80	$\Delta ku80::pyrGAF$	FGSCA1151
Ku80pyrG	$\Delta ku80::pyrGAF pyrG$	Our laboratory
$\Delta mpkC$	$\Delta mpkC::prtA$	Hagiwara et al. (2014)
$\Delta sakA$	$\Delta sakA::hph$	Hagiwara et al. (2014)
$\Delta mpkC \Delta sakA$	$\Delta mpkC::prtA \Delta sakA::hph$	Bruder Nascimento et al. (2016)
$\Delta mpkC::mpkC^+$	$\Delta mpkC::mpkC^+::prtA$	Bruder Nascimento et al. (2016)
$\Delta sakA::sakA^+$	$\Delta sakA::sakA^+::prtA$	Bruder Nascimento et al. (2016)
$\Delta mpkA$	$\Delta mpkA::hph$	Valiante et al. (2009)
$\Delta mpkA::mpkA^+$	$\Delta mpkA::sakA^+::prtA$	Valiante et al. (2009)
$\Delta sitA$	$\Delta sitA::pyrG^+$	Bom et al., 2015
$\Delta sitA::sitA^+$	$\Delta sitA::sitA^+::hph$	Bom et al., 2015
$\Delta ptcB$	$\Delta ptcB::pyrG^+$	Winkelströter et al. (2015a)
$\Delta ptcB::ptcB^+$	$\Delta ptcB::ptcB^+::hph$	Winkelströter et al. (2015a)
$\Delta pphA$	$\Delta pphA::pyrG^+$	Winkelströter et al. (2015b)
$\Delta pphA::pphA^+$	$\Delta pphA::pphA^+::hph$	Winkelströter et al. (2015b)

uridine) and liquid YG or YUU medium of the same composition (but without agar). A modified minimal medium (MM: 1% (w/v) glucose, original high nitrate salts, trace elements, 2% (w/v) agar, pH 6.5) was also applied. Trace elements, vitamins, and nitrate salts were described by (Käfer, 1977).

Phenotypic assays

The phenotypes of the deletion mutants were evaluated by radial growth at different temperatures, in the presence or absence of stressing agents. Five μ l of a concentration of 2×10^7 for the wild-type and mutant strains were spotted on different growth media and grown for 96 h at 37 °C.

The adherence to fibronectin was assessed according to Gravelat et al. (2010) with modifications. Briefly, wells from a sterile 96-well flat-bottom polystyrene plate were previously coated with 250 μ l of fibronectin 0.01 mg/mL (Sigma-Aldrich) diluted in PBS. The plates were incubated at 37 °C for 16 h and washed twice with PBS. Further, 1×10^4 conidia were inoculated into 200 μ l of liquid MM in each well, incubated for additional 24 h at 37 °C and washed 3 times with sterile PBS (NaCl, 137 mM; KCl, 2.7 mM; Na₂HPO₄, 10 mM; and KH₂PO₄, 1.8 mM). The adherent cells were stained with 200 μ l of 0.5% crystal violet solution for 5 min at room temperature, exhaustively washed with PBS and air dried. Finally, the crystal violet was eluted from the wells using 200 μ l of 100% ethanol, and the absorbance was measured at 590 nm. The capacity of adhesion to plastic surface was performed similarly to described above except by the fact the wells were not covered with fibronectin (Mowat et al., 2007). All the experiments were performed at least three times.

Staining for dextrin-1, chitin, and other carbohydrates

This procedure was performed as described by Graham et al. (2006), Winkelströter et al. (2015a,b). Briefly, *A. fumigatus* conidia (2×10^3) were grown in 200 μ l of MM for 16 h at 37 °C. The culture medium was removed and the germlings were UV-irradiated (600,000 μ J). The germlings were washed with PBS and 200 μ l blocking solution (goat serum 2% (w/v), BSA 1% (w/v), 0.1% (v/v) Triton X-100, 0.05% (v/v) Tween 20, 0.05% (v/v) sodium azide and 0.01 M PBS) were added and incubated for 30 min at room temperature. For dextrin staining, 0.2 μ g/mL of Fc-h-dextrin-hFc were added and incubated for 1 h at room temperature, followed by adding 1:1000 DyLight 594-conjugated, goat anti-human IgG1 and incubated for 1 h at room temperature (Graham et al., 2006). Germlings were washed with PBS and fluorescence was read at 587 nm excitation and 615 nm emission. For chitin staining, 200 μ l of a PBS solution with 10 μ g/mL of CFW were added to UV-irradiated germlings, incubated for 5 min at room temperature, washed three times with PBS and fluorescence was read at 380 nm excitation and 450 nm emission. For GAG, GlcN, and mannose staining, 200 μ l of PBS with 0.1 mg/ml of SBA-FITC (Glycine max Soybean lectin SBA-FITC bioworld cat# 21761024-2), WGA (Lectin-FITC L4895, Sigma) or ConA (Concanavalin A C7642, Sigma), respectively, were added to UV-irradiated germlings. The germlings were incubated for 1 h at room temperature, washed with PBS, and fluorescence was read at 492 nm excitation and 517 nm emission. All the experiments were performed with 12 repetitions and fluorescence was read in a microtiter plate reader SpectraMax i3 (Molecular Devices).

SYTO9 fluorescence assay

Adhesion of *A. fumigatus* conidia to polystyrene surface was assessed by SYTO9 fluorescence assay (Honraet et al., 2005). Briefly, *A. fumigatus* conidia (1×10^5) were grown in RPMI media in black 96 well polystyrene plate for 90 min at 37 °C. The culture medium was removed and any non-adherent cells were washed off with distilled water. Subsequently, adherent cells were stained with 100 μ l of SYTO 9 dye

(10 μ M) for 15 min at 37 °C. Fluorescence was then measured using a microtiter plate reader (FLUOstar omega, BMG Labtech) at an excitation and emission wavelength of 485 nm and 505 nm, respectively.

Scanning electron microscopy (SEM)

Standardized conidia of Δ pphA and its parental strain were inoculated in RPMI medium onto Thermanox coverslips (13 mm) within a 24-well tissue culture plate. After 24 h incubation at 37 °C, biofilms were processed and imaged as previously described (Erlandsen et al. (2004a,b). Briefly, the biofilms were washed in PBS and fixed in 2% paraformaldehyde, 2% glutaraldehyde, and 0.15% (w/v) alcian blue in 0.15 M sodium cacodylate (pH 7.4). The biofilms were sputter coated with gold and viewed under a JEOL JSM-6400 scanning electron microscope in high vacuum mode at 10 kV.

Transmission electron microscopy (TEM)

Preparation of samples was as previously described (Walker et al., 2008) with the following modifications. Briefly, cells were collected and the pellets were fixed with 2.5% (v/v) glutaraldehyde in 0.1 M sodium phosphate buffer (pH 7.3) for 24 h at 4 °C. Samples were encapsulated in 3% (w/v) low melting point agarose prior to processing to Spurr resin following a 24 h schedule on a Lynx tissue processor (secondary 1% OsO₄ fixation, 1% Uranyl acetate contrasting, ethanol dehydration and infiltration with acetone/Spurr resin). Additional infiltration was provided under vacuum at 60 °C before embedding in TAAB capsules and polymerising at 60 °C for 48 h. Semi-thin (0.5 μ m) survey sections were stained with toluidine blue to identify areas of best cell density. Ultrathin sections (60 nm) were prepared using a Diatome diamond knife on a Leica UC6 ultramicrotome, and stained with uranyl acetate and lead citrate for examination with a JEM-1400Plus transmission electron microscope (JEOL (U.K.) LTD, Hertfordshire, UK) and imaging with an AMT UltraVUE camera and AMT Image Capture Engine V602 software (Deben UK Limited, Suffolk, UK).

Dictyostelium discoideum phagocytosis assay

D. discoideum was grown in Petri dishes for 16 h at 22 °C in HL5 (axenic medium) supplemented with streptomycin sulfate (300 μ g/mL). Subsequently, the plates were washed and the supernatant was centrifuged for 5 min at 1500 rpm, the supernatant was discarded, and the pelleted cells were resuspended in 3 mL of HL5 medium. The density of *D. discoideum* cells was determined by counting the cells in a hemocytometer as described at <http://dictybase.org/>. As described by Hillmann et al. (2015), 10 conidia per cell of *D. discoideum* (different multiplicities of infection, MOIs) were used for the co-incubation. *D. discoideum* (10^5) cells and *A. fumigatus* conidia (10^6) were added to a six-well plate and the plate was incubated at 22 °C for 5 h, after which time the 50 *D. discoideum* cells were counted and the number of phagocytosed conidia evaluated.

Murine model of pulmonary aspergillosis

The murine model of pulmonary aspergillosis was performed according to Dinamarco et al. (2012). Outbred female mice (BALB/c strain; body weight, 20–22 g) were housed in vented cages containing 5 animals. Mice were immunosuppressed with cyclophosphamide at a concentration of 150 mg per kg of body weight, which was administered intraperitoneally on days –4, –1, and 2 prior to and post infection (day 0). Hydrocortisone acetate (200 mg/kg) was injected subcutaneously on day –3. The *A. fumigatus* conidia used for inoculation were grown on *Aspergillus* complete YAG for 2 days prior to infection. Fresh conidia were harvested in PBS and filtered through a Miracloth (Calbiochem). Conidial suspensions were spun for 5 min at 3000 $\times$ g, washed three times with PBS, counted using a hemocytometer, then

resuspended at a concentration of 5.0×10^6 conidia/mL. Viability counts for the administered inoculum were determined, following serial dilution, and plating on *Aspergillus* YGA, and the conidia were grown at 37 °C. Mice were anesthetized by halothane inhalation and infected by intranasal instillation of 1.0×10^5 conidia in 20 µL of PBS. As a negative control, a group of 5 mice received PBS only. Mice were weighed every 24 h from the day of infection and visually inspected twice daily. In the majority of cases, the endpoint for survival experimentation was identified when a 20% reduction in body weight was recorded, at which time the mice were sacrificed. The statistical significance of comparative survival values was calculated using log rank analysis using the Prism statistical analysis package. Additionally, at 3 days post infection, 2 mice per strain were sacrificed and the lungs were removed, fixed, and processed for histological analysis.

Lung histopathology and fungal burden

After sacrifice, the mice lungs were removed and fixed for 24 h in 3.7% (v/v) formaldehyde-PBS. Samples were washed several times in 70% (v/v) alcohol before dehydration in a series of alcohol solutions of increasing concentrations. Finally, the samples were diafanized in xylol and embedded in paraffin. For each sample, sequential 5-µm-thick sections were collected on glass slides and stained with Gomori methenamine silver (GMS) or hematoxylin and eosin (HE) stain following standard protocols (Greenberger, 2002). Briefly, sections were deparaffinized, oxidized with 4% chromic acid, stained with methenamine silver solution, and counterstained with hematoxylin. Tissue sections were also stained with hematoxylin and eosin for histological examination to determine lung damage. All stained slides were immediately washed, preserved in mounting medium, and sealed with a coverslip. Microscopic analyses were performed using an Axioplan 2 imaging microscope (Carl Zeiss) at the stated magnifications under bright-field conditions.

To investigate fungal burden in the lungs, mice were infected as described previously, but with a higher inoculum of 1×10^6 conidia/20 µL. A higher inoculum, in comparison to the survival experiments, was used to increase fungal DNA detection. Animals were sacrificed 72 h post infection, and both lungs were harvested and immediately frozen in liquid nitrogen. Samples were homogenized by vortexing with glass beads for 10 min, and DNA was extracted via the phenol–chloroform method. DNA quantity and quality were assessed using a NanoDrop 2000 spectrophotometer (Thermo Scientific). At least 500 ng of total DNA from each sample was used for quantitative real-time PCR. A primer and a Lux probe (Invitrogen) were used to amplify the 18S rRNA region of *A. fumigatus* and an intronic region of mouse GAPDH (glyceraldehyde-3-phosphate dehydrogenase). Six-point standard curves were calculated using serial dilutions of gDNA from all the *A. fumigatus* strains used and the uninfected mouse lung. Fungal and mouse DNA quantities were obtained from the threshold cycle (CT) values from an appropriate standard curve. Fungal burden was determined as the ratio between picograms of fungal and micrograms of mouse DNA.

Determination of TNF-α levels

For cytokine determination, bone marrow derived macrophages (BMDMs) from C57BL/6 mice were prepared as previously described (Marim et al., 2010). Briefly, bone marrow cells from femurs of adult mice were cultured for 6 days in RPMI 1640, containing 20% (v/v) fetal bovine serum (FBS) and 30% (v/v) L-929 cell conditioned media (LCCM). Macrophages (5.0×10^5) were plated in 48-well plates for 16 h at 37 °C, 5% (v/v) CO₂ in RPMI 140 media containing 10% (v/v) FBS and 5% (v/v) of LCCM. For fungal infection, strains were cultured for 18 h up to a hyphal stage at a density of 2×10^4 per well, UV-irradiated and used to stimulate the macrophages. The cells were centrifuged to synchronize the infection and allowed to infect for 18 h. The

supernatant was collected and the cytokine was measured by enzyme-linked immunosorbent assay (ELISA) with a mouse TNF-α kit (R&D Quantikine ELISA) according to the manufacturer's instructions.

Statistical analysis

All data, except the survival curves, were analyzed using t-test (Prism, GraphPad) with significance levels of **p < 0.01 and ***p < 0.001. The statistical significance of comparative survival values was calculated using log rank analysis using the Prism statistical analysis package.

Results

MAPKs are important for biofilm formation

Initially, we assessed the adhesive capacity of the respective mutants, which is the initial step in the foundation of biofilm formation, to assess the impact of loss of the MAP kinases on the adherence to plastic or fibronectin. Germlings of the $\Delta mpkA$, $\Delta sakA$, and $\Delta mpkC \Delta sakA$ mutants displayed reduced adherence to polystyrene plates ranging from 70 to 75% when compared to the wild type. The adherence to fibronectin-coated plates was reduced ranging from 50 to 60% when comparing the mutants $\Delta mpkA$, $\Delta sakA$, and $\Delta mpkC \Delta sakA$ to the wild type strain (Fig. 1A and B). Scanning electron microscopy (SEM) of the mycelia revealed that $\Delta sakA$, $\Delta mpkC$, $\Delta mpkC \Delta sakA$, and $\Delta mpkA$ influenced to different extents the cell surface and the ECM during biofilm formation (Fig. 2). The surface of the MAP kinase null mutants appeared to have reduced ECM, which was in stark contrast to the wild-type strain (Fig. 2). This decreased adhesion and ECM formation could be due to alterations in the composition of the carbohydrates present in the cell wall. Thus, cell wall stains and lectins were used to identify differences in the content or exposure of different carbohydrates on the surface of the fungal cell wall, in both the wild-type and MAP kinase mutants. These included: (i) SBA (Soybean Agglutinin)-FITC (preferentially binds to oligosaccharide structures with terminal α- or β-linked N-acetylgalactosamine (GalNAc), and to a lesser extent, galactose residues – important for recognizing galactosaminogalactan, GAG), (ii) WGA (Wheat Germ Agglutinin)-FITC [recognizing surface exposed N-acetyl glucosamine (GlcNAc)], (iii) ConA (Concanavalin A)-FITC (recognizes α-linked mannose), (iv) soluble dectin-1 staining (recognizing β-glucans), and (v) CFW (recognizing chitin). When the mutants were compared with the wild-type and the corresponding complemented strains (Fig. 3A–H), for $\Delta mpkA$ we observed 50% less N-acetyl glucosamine (Fig. 3A), 50% less GAG (Fig. 3C), 50% less mannose (Fig. 3E), 40% less chitin (Fig. 3G), and 40% more β-glucan (Fig. 3H). In contrast there was 50 % more GAG in the $\Delta mpkC$, $\Delta sakA$, $\Delta mpkC \Delta sakA$ mutants (Fig. 3D). Previously, we have shown that the intensity of CFW staining per fungal area was 30% higher in $\Delta mpkC$ mutant than wild-type and complemented strains, while $\Delta sakA$ and $\Delta mpkC \Delta sakA$ were 20% lower than wild-type and complemented strains (Bruder Nascimento et al., 2016). Moreover, the $\Delta mpkC$, $\Delta sakA$ and $\Delta mpkC \Delta sakA$ mutant strains were shown to have more abundant β-glucans than the wild-type and complemented strains. The intensity of dectin-1 staining per fungal area was 50, 40, and 30% higher in the $\Delta mpkC$, $\Delta sakA$ and $\Delta mpkC \Delta sakA$ mutants, respectively compared with wild-type strain (Bruder Nascimento et al., 2016).

Next step, we characterized the distribution of WGA-FITC, SBA-FITC, and ConA-FITC binding (Fig. 4A–C) to phosphatase null mutants previously shown to be involved in modulating MpkA or SakA phosphorylation, $\Delta sitA$, $\Delta ptcB$, and $\Delta pphA$ (Winkelströter et al., 2015a,b; Bom et al., 2015). A number of differences were observed when the mutants were compared with the wild-type and the corresponding complemented strains (Fig. 4A–H). We have observed 40% more WGA-FITC binding to $\Delta pphA$ and fourfold less to $\Delta ptcB$ (Fig. 4A), 50–60% less SBA-FITC binding to $\Delta pphA$ and $\Delta sitA$ (Fig. 4B). In addition there was

Fig. 1. The adhesion of *A. fumigatus* wild-type and MAP kinase mutants. (A and B) Fungal adhesion to plastic or fibronectin was measured by crystal violet (CV) assay. The results are the average of three experiments $\pm$ standard deviation (* = $p < 0.001$ by *t*-tests).

20% more mannose in $\Delta pphA$ and 50% and 10% less mannose in $\Delta sitA$ and $\Delta ptcB$, respectively (Fig. 4C). Again, we have previously shown the intensity of dectin-1 and CFW staining per fungal area was twofold and 25% higher in the $\Delta ptcB$ mutant compared to wild-type strain (Winkelströter et al., 2015a). The intensity of dectin-1 and CFW staining per fungal area was 40% and 20% higher in $\Delta sitA$ mutant than

wild-type strain (Bom et al., 2015). All these modifications in the cell wall increase the MAP kinase, $\Delta ptcB$, and $\Delta sitA$ mutants conidial phagocytosis by *Dictyostelium discoideum* in about two to threefold when compared to the wild-type and complemented strains (Fig. 5A–F).

Collectively, our results demonstrate that MAP kinases and phosphatases that affect MpkA and SakA phosphorylation, influence the

Fig. 2. Scanning Electron Microscopy for the wild-type and MAP kinase null mutant strains. In the upper, middle, and lower panels 1000, 5000, and 10,000 times magnification were used respectively. The arrow heads indicate the hypha surface.

Fig. 3. Carbohydrate content and exposure on the surface of the wild-type and MAP kinase mutants. (A and B) WGA-FITC staining, (C and D) SBA-FITC staining, (E and F) ConA-FITC staining, (G) CFW staining, and (H) Dectin staining. The results are the average of twelve repetitions $\pm$ standard deviation (*** = $p < 0.001$ and ** = $p < 0.01$ by t -tests).

carbohydrate content and exposure at the cell wall, thus impacting adhesion, biofilm formation and phagocytosis by *Dictyostelium*.

PphA is a novel phosphatase that affects the CWI pathway

We decided to investigate in more detail the $\Delta pphA$ phosphatase mutant. The $\Delta pphA$ mutant has less chitin and more β -1-3 glucan than

the wild-type and complemented strains. The intensity of CFW and dectin-1 staining were 80% lower and 30% higher in the $\Delta pphA$ mutant, respectively compared with wild-type strain (Fig. 6A and B). This mutant is more sensitive to Congo Red (CR) and CFW than the wild-type and complemented strains (Fig. 6C and D). It shows sensitivity to lower caspofungin concentrations such as 0.125 and 0.5 μ g/ml, but it preserved the caspofungin paradoxical effect or trailing, (i.e. the escape of

Fig. 4. Carbohydrate content and exposure on the surface of the wild-type and phosphatase mutants. (A) WGA-FITC staining, (B) SBA-FITC staining, and (C) ConA-FITC staining. The results are the average of twelve repetitions $\pm$ standard deviation (***) = $p < 0.001$ and ** = $p < 0.01$ by t -tests).

A. fumigatus from caspofungin inhibition at concentrations above the minimal inhibitory concentration; Chen et al., 2011; Fig. 6E). Possible modifications in the cell wall decrease $\Delta pphA$ conidia phagocytosis by about 30% when compared to the wild-type and complemented strains (Fig. 6F). Germlings of $\Delta pphA$ showed comparable % adherence to polystyrene plates and fibronectin-coated plates (Fig. 6G). Furthermore, a sensitive SYTO 9 fluorescence based assay showed a significant ($p < 0.05$) reduction of about 4-fold in adherence with $pphA$ compared to the wild type strain (Fig. 6H).

Additional evidence for a role of PphA in biofilm formation and in the organization of the cell wall was provided by SEM and TEM (Transmission Electron Microscopy) analyses (Fig. 7A). SEM of the mycelia revealed that PphA influenced the cell surface and the ECM during biofilm formation (Fig. 7A). The surface of the $\Delta pphA$ appeared

smooth, which was in stark contrast to the wild-type strain (Fig. 7A). TEM analysis showed that untreated $\Delta pphA$ germlings grown in MM have about 25% thicker cell walls compared to the wild-type (Fig. 7B). Further exposure of the $\Delta pphA$ germlings to CFW decreased the cell wall thickness about 30% (Fig. 7B). In contrast, when wild-type strain was exposed to CFW and CR, it had about 50% increased cell wall thickness (Fig. 7B).

We also investigated if PphA was involved in the MpkA and/or SakA pathways in *A. fumigatus*, by determining the phosphorylation state of MpkA and SakA in the presence and absence of CR and sorbitol stresses. In both stressing conditions, the phosphorylation levels of MpkA and SakA were similar in both the wild-type and $\Delta pphA$ mutant strains (Supplementary Fig. S1). Calphostin C, chelerythrine, and cercosporamide have been used as protein kinase C inhibitors in mammals and in

Fig. 5. Phagocytosis of MAP kinase and phosphatase null mutants by *Dictyostelium discoideum*. (A–F) Conidia from the wild-type and MAP kinase and phosphatase null mutants were exposed to *D. discoideum* and the total number of phagocytosed conidia evaluated. The results are the average of three repetitions with $\pm$ standard deviation. The asterisks indicate $p < 0.001$.

fungi (Arpaia et al., 1999; da Rocha et al., 2002; Herbert et al., 1990; Jarvis and Grant, 1999; Juvvadi et al., 2007; Sussman et al., 2004). We hypothesize if *ApphA* is modulating the protein kinase C activity, the susceptibility status of *ApphA* to these inhibitors would be altered. The *ApphA* and wild-type growth was similarly inhibited by calphostin C, cercosporamide, and chelerythrine (Supplementary Fig. S2).

Taken together these results strongly indicate that PphA is important for the *A. fumigatus* cell wall integrity pathway. However, PphA is not modulating either MpkA, SakA or Protein kinase C activity.

PphA is important for *A. fumigatus* virulence

The importance of PphA for *A. fumigatus* pathogenicity was evaluated in a neutropenic murine model of invasive pulmonary aspergillosis (Fig. 8A). There are no statistical differences among the wild-type and *ApphA::pphA*⁺ strains (using two different statistical tests, Log-rank Mantel-Cox and Gehan-Breslow-Wilcoxon). Wild-type infection resulted in 100% mortality 12 days post-infection, while *ApphA::pphA*⁺ infection resulted in a significant mortality rate of 100% after 13 days post-infection (Fig. 8A). The *ApphA* infection resulted in the greatest reduction in mortality rate, with only 25% of mice dying after 15 days post-infection (Fig. 8A, $p < 0.0025$ and $p < 0.0041$ for the comparison between the wild-type and the *ApphA* mutant, Log-rank Mantel-Cox and Gehan-Breslow-Wilcoxon tests, respectively).

Fungal burden was measured by qPCR, *ApphA* had only 15% of the fungal burden of the wild type and complemented strains (Fig. 8B). Histopathological examination revealed that at 72 h post-infection the lungs of mice infected with the wild-type or complemented strains contained multiple foci of invasive hyphal growth; the majority of the disease seems peri-bronchiole with some extension into the lung parenchyma (Fig. 8C). In contrast, *ApphA* infections revealed inflammatory infiltrates in bronchioles, with some containing poorly germinated or non-germinated conidia (Fig. 8C). Taken together, these results clearly demonstrate that PphA plays an important role in *A. fumigatus* virulence.

The impaired *ApphA* CWI together with the dramatic attenuation in virulence could contribute to an altered immune response. Subsequently, the cytokine Tumour Necrosis Factor alpha (TNF- α) levels released from bone marrow derived macrophages (BMDMs) after co-incubation with *A. fumigatus* conidia, germlings, and hyphae were investigated. TNF- α is an important inflammatory mediator secreted by

macrophages when exposed to *A. fumigatus* (Hayashi et al., 2005; Taramelli et al., 1996) and it has shown to be an important signal in the initiation and maintenance of innate immunity in different animal models of pneumonia (Gosselin et al., 1995; Laichalk et al., 1996). The innate immunity represents the principal pathway by which *A. fumigatus* is cleared from the lung. This innate response consists specially by macrophages, which represent the first line of defense against conidia entering the alveolus, and recruited neutrophils (Schaffner et al., 1982). Bone Marrow Derived Macrophages (BMDMs) co-cultured with wild-type, *ApphA* *ApphA::pphA*⁺ showed a comparable TNF- α production when exposed to germlings and hypha (Fig. 8D). However, the *ApphA* conidia showed about 7-fold TNF- α induction than conidia from the wild-type or complemented strains (Fig. 8D). These results suggest that the effect caused by the absence of PphA on conidia is important for macrophage recognition and inducing inflammatory responses.

Discussion

A. fumigatus represents the most common airborne human pathogenic fungus in the world. Humans inhale hundreds of *A. fumigatus* asexual conidia daily and they cause a number of diseases, ranging from simple allergies to lethal invasive aspergillosis (IA) in immunocompromised patients (Latgé & Steinbach, 2009). Although *A. fumigatus* virulence has been investigated in many studies in recent years (Carberry et al., 2012; Chotirmall et al., 2014; Ding et al., 2014; Grahl et al., 2012; Haas, 2014; Hartmann et al., 2011; Moore, 2013; Scharf et al., 2012; Schrettl & Haas, 2011; Wezensky & Cramer, 2011), the signal transduction pathways that are activated during the different clinical forms of the disease are still unclear. Recently, aiming to investigate the signal transduction pathways that make *A. fumigatus* a successful pathogen, we have characterized the role of Mitogen-Activated Protein (MAP)-kinases and phosphatases in the adaptive response of this fungus to different types of stresses (Bom et al., 2015; Bruder Nascimento et al., 2016; Winkelströter et al., 2015a,b). *A. fumigatus* has four MAPKs: MpkA, which regulates the cell wall integrity (CWI) signaling and also has a role in the oxidative stress response (Valiante et al., 2015), MpkC and SakA, the mitogen-activated protein kinases of the high-osmolarity glycerol (HOG) pathway, that are involved in response to osmotic and oxidative stress, play a role in carbon source utilization and caspofungin adaptation (Altwasser et al., 2015; Bruder Nascimento et al., 2016; Du et al., 2006; Reyes et al., 2006; Posas et al.,

Fig. 6. The PphA null mutant has impaired cell wall integrity. (A and B) The *A. fumigatus* $\Delta pphA$ mutant has decreased chitin and increased β -1,3-glucan. The results are the average of twelve repetitions $\pm$ standard deviation ($^* = p < 0.001$ by *t*-tests). (C–E) The wild-type, $\Delta pphA$, and $\Delta pphA::pphA^+$ strains were grown for 120 h at 37 °C in MM in the presence or absence of different concentrations of Congo Red (CR), Calcofluor White (CFW) and Caspofungin. These experiments were performed by measuring radial diameter in triplicate, and the results are displayed as mean values with standard errors ($^* = p < 0.001$ by *t*-tests). (F) Phagocytosis of *A. fumigatus* conidia by *Dictyostelium discoideum*. A ratio of 10⁶ amoeba cells to 10⁷ conidia was used in each experiment and they were incubated for 5 h at 22 °C. Fifty amoeba cells were evaluated for phagocytosis. The results are the average of three independent experiments $\pm$ standard deviation. (G) The adhesion of *A. fumigatus* wild-type, $\Delta pphA$, and $\Delta pphA::pphA^+$ mutants. Fungal adhesion as measured by crystal violet (CV) assay on the adherence to plastic or fibronectin was examined. (H) SYTO9 adhesion assay with the wild-type and $\Delta pphA$ mutant strains. The results are displayed as mean values with standard errors ($^* = p < 0.001$ by *t*-tests).

1996), and MpkB, homologous to yeast Fus3, still uncharacterized (Elion et al., 1990). Here, we explored the impact of loss of the MAP kinases and phosphatases on the adhesion properties and biofilm formation in *A. fumigatus*. Such *A. fumigatus* biofilms are formed *in vitro* during static growth on a solid substratum (Beauvais et al., 2007) and in mouse lung with IA and in human lung aspergilloma (Loussert et al., 2010). Germlings of the $\Delta mpkA$, $\Delta sakA$, and the double $\Delta mpkC \Delta sakA$ showed reduced adherence. Scanning electron microscopy for the wild-type and the mutant strains demonstrated that all the MAP kinase null mutants displayed altered cell surfaces and have altered ECM

production during biofilm formation. In *C. albicans*, the CWI MAP kinase Mkc1p, the homologue of the *A. fumigatus* MpkA, is activated in a contact-dependent manner and it was shown that this kinase is necessary for normal biofilm development and invasive hyphal growth (Kumamoto, 2005). Another *C. albicans* kinase, Pkh3, is crucial for cell substrate adherence (Fanning et al., 2012), an essential stage of biofilm establishment (Finkel & Mitchell, 2011). Six master transcriptional regulators control *C. albicans* biofilm development at the genetic level. These six proteins (Efg1, Tec1, Bcr1, Ndt80, Brg1, and Rob1) are required for biofilm development both *in vivo* and *in vitro* (Nobile et al.,

Fig. 7. PphA plays a role in biofilm formation and in the organization of the cell wall as demonstrated by SEM and TEM analyses. (A) SEM for the wild-type and $\Delta pphA$ mutant strains. The magnification is 1000 and 4000 times. (B) TEM for *A. fumigatus* wild-type and $\Delta pphA$ mutant strains. Germings were grown in the absence or presence of CFW (Calcofluor White) or Congo Red. TEM analysis (bars, 100 nm). Cell wall thickness (μm) of fifty sections of different germings were measured. The results are expressed as average $\pm$ standard deviation (* = $p < 0.001$ by t -tests).

2012) and regulate the expression of around 1000 target genes. Bcr1, a transcriptional master regulator of biofilm production, and its cell wall protein targets Hwp1, Als1 and Als3, are needed for adherence during biofilm establishment (Chandra et al., 2001; Nobile et al., 2006a,b, 2008; Nobile and Mitchell, 2005; Zhao et al., 2006). The distinct phases of biofilm production in *C. albicans* are regulated by different signaling pathways: yeast to hyphae transition involves Ras/cAMP/PKA (Hogan & Sundstrom, 2009); the induction of pseudohyphal growth is controlled by Cek1 MAPK (Srinivasa et al., 2012); and the calcium signaling pathways are important for morphogenesis (Sanglard et al.,

Fig. 8. *A. fumigatus* PphA contributes to virulence in neutropenic mice. (A) Comparative analysis of wild-type and mutant strains in a neutropenic murine model of pulmonary aspergillosis. Mice in groups of 10 per strain were infected intranasally with a 20 μl suspension of conidiospores at a dose of 10^5 . (B) Fungal burden results, determined by qPCR 72 h post-infection, were expressed based on 18S rRNA gene of *A. fumigatus* divided by the results of an intronic region of the mouse GAPDH gene. (C) Histological analysis of infection murine lung was performed 72 h after infection. Lower panels show increased magnification of the lined area from upper figure. Arrows indicate germings. (D) TNF- α secretion from bone marrow-derived macrophages (BMDMs). The $\Delta pphA$ conidia triggered significantly increased release of TNF- α from BMDMs compared to wild-type and the reconstituted strain at 0 h time point. BMDMs from C57BL/6 mice were infected with *A. fumigatus* conidia, germings, or hyphae for up to 18 h and the supernatant of cells was collected to measure the TNF- α levels by ELISA. Data show average $\pm$ standard deviation and * $p \leq 0.005$ compared to the wild-type and the complemented strain.

2003). For example, the inactivation of the transcription regulator Efg1p (Ras/cAMP/PKA pathway) or Cph1p (MAP kinase pathway) can block hyphal growth and biofilm formation (Lo et al., 1997). In addition, it was demonstrated that filamentous growth was inhibited in cells deficient in Cdc35p showing the role of the adenylyl cyclase pathway in hyphal development (Rocha et al., 2001). A recent study involving a collection of 63 *C. albicans* protein kinase mutants showed that 38 displayed some degree of biofilm defect. Network analysis demonstrated functional redundancy of the proteins involved in some processes, including MAP kinases, since individual components are not required for biofilm formation (Konstantinidou & Morrissey, 2015). In this work we presented that the single deletion of Map kinases in *A. fumigatus* caused impaired biofilm formation. However, it is important to note that some changes in growth conditions for biofilm formation, such as surface support and characteristics of the medium, may produce different results.

We decided to investigate if this decreased biofilm formation could be due to alterations in the exposure of the polysaccharides present in the cell wall. The *A. fumigatus* cell wall contains different types of polysaccharides, such as: β -(1,3)-/ β -(1,4)-glucan, chitin and galactomannan, which are covalently bound to branched β -(1,3)-glucan and α -(1,3)-glucan, which maintain the stability of the cell wall through its adhesive properties (Beauvais et al., 2013). The cell wall of the conidia that are inhaled are different compared to vegetative mycelium. The conidial cell wall has an outer layer composed of melanin and rodlets. These components display hydrophobic properties and prevent immune recognition of airborne fungal spores (Aimanianda et al., 2009; Aimanianda and Latgé, 2010; Carrion et al., 2013). In contrast to this, the external layer of the hypha presents galactosaminogalactan (GAG), an important *A. fumigatus* virulence factor (Beauvais et al., 2014). The *A. fumigatus* biofilm ECM is mainly composed of galactomannan, GAG, α -1,3 glucan, proteins, polyols, melanin and eDNA (Rajendran et al., 2013). *A. fumigatus* $\Delta mpkA$ germlings have reduced exposure of N-acetyl glucosamine, GAG, mannose and chitin, and increased β -1,3-glucan. The cell wall of *A. fumigatus* has pathogen-associated molecular patterns (PAMPs) that are identified by pattern recognition receptors (PRRs) on innate immune cells of the host. β -1,3-glucan acts as a PAMP and is recognized by the C-type lectin receptor (CLR) dectin 1 and this process is essential for initial host defense mechanisms in the respiratory tract (Brown & Gordon, 2001; Gessner et al., 2012; Werner et al., 2009). The cell wall component GAG, which is secreted during hyphal growth and is present in the *A. fumigatus* biofilm, mediates adherence and has a role attenuating the host immune response through inducing interleukin-1 receptor antagonist and also by masking β -1,3 glucan (Gravelat et al., 2013; Gressnigt et al., 2014). Thus, our results show that the $\Delta mpkA$ mutant displays changes in exposure of β -1,3-glucan and GAG, the two most important polysaccharides involved in the recognition of the *A. fumigatus* during host infection.

Recently our group showed that the $\Delta sakA$ and the double $\Delta mpkC \Delta sakA$ mutants were more sensitive to cell wall damaging agents (Bruder Nascimento et al., 2016). The distribution of β -1,3-glucan and chitin content on the cell surface of these mutants was altered when compared to the wild type. The $\Delta sakA$, $\Delta mpkC$ and $\Delta mpkC \Delta sakA$ mutant strains have more abundant β -glucans than the wild-type, and the chitin content was higher in the $\Delta mpkC$ and lower in $\Delta sakA$ and $\Delta mpkC \Delta sakA$. Moreover, the single mutants $\Delta sakA$ and $\Delta mpkC$ were virulent in a low dose murine infection model and the double $\Delta mpkC \Delta sakA$ had highly attenuated virulence in the same model of infection (Bruder Nascimento et al., 2016). In this work we showed that $\Delta sakA$, $\Delta mpkC$ and $\Delta mpkC \Delta sakA$ displayed reduced exposure of GAG in the cell wall. All these results suggest that the mitogen-activated protein kinases of the HOG pathway play an important role in the CWI pathway and biofilm formation in *A. fumigatus*. However, which transcriptional factors are activated downstream of the SakA and MpkC during the cell wall stress response and biofilm formation is still unknown. Previously, our group showed that deletion of the SitA and PtcB phosphatases

activated the cell wall integrity pathway in *A. fumigatus* and this event was linked to suppression of ECM production and biofilm formation (Bom et al., 2015; Winkelströter et al., 2015a). PtcB is involved in the HOG pathway regulating the expression of osmo-dependent genes and phosphorylation of SakA. Moreover, $\Delta sitA$ and $\Delta ptcB$ had increased exposure of β -1-3 glucan and chitin in the cell wall and both these mutant strains were avirulent in a murine model of invasive pulmonary aspergillosis. The results presented here showed that $\Delta sitA$ and $\Delta ptcB$ have reduced exposure of mannose content in the cell wall and chitin and GAG were less abundant in the $\Delta ptcB$ and $\Delta sitA$, respectively. All these modifications in the cell wall increase the MAP kinase, *ptcB*, and *sitA* mutant conidia phagocytosis by *D. discoideum*.

Here we investigated in detail the phosphatase-encoding gene PphA. The *A. fumigatus* gene *pphA* codes for a Ser/Thr protein phosphatase PphA member of the PPP4 phosphatase family, which is homologous to yeast PPH3. In *S. cerevisiae*, PPH3 forms a phosphatase complex Pph3/Psy2 and it is involved in the regulation of Non-Homologous End-Joining (NHEJ) Pathway. Pph3/Psy2 complex controls the cell cycle progression dephosphorylating the key protein Rad53p and allowing cell cycle to continue (Omidi et al., 2014). Furthermore, Pph3/Psy2 targets the glucose signal protein Mth1 and this activity is required for the dephosphorylation of the downstream transcriptional repressor Rgt1 under glucose removal, an essential event in the repression of *HXT* genes, which encode glucose transporters. Mth1 and its associated corepressor Rgt1 are dephosphorylated by Pph3/Psy2, but this complex does not dephosphorylate Rgt1 at sites correlated with inactivation. The protein kinase A (PKA), the most important protein kinase activated in response to glucose, phosphorylates Mth1 via putative Pph3/Psy2 dephosphorylation sites *in vivo*. *S. cerevisiae* Pph3/Psy2 phosphatase negatively regulates Mth1 phosphorylation by protein kinase A (PKA) (Ma et al., 2013). In *C. albicans*, Pph3 dephosphorylates Rad53, a DNA checkpoint kinase that regulates effectors which execute the stress response to DNA damage (Omidi et al., 2014). The *A. fumigatus* PphA phosphatase has a notable influence on the composition of the carbohydrates present in the cell wall. $\Delta pphA$ germlings have increased exposure of β -1,3 glucan, mannose and N-acetyl glucosamine, and the GAG and chitin content are reduced. Unexpectedly, when confronted with *D. discoideum*, the $\Delta pphA$ deletion mutant conidia were less frequently phagocytosed than those of the parental wild-type strain.

The *A. fumigatus* $\Delta pphA$ strain was more sensitive to Congo Red (CR) and CFW than the wild type strain, and we also have observed its sensitivity to lower caspofungin concentrations. PphA also had an important impact on the cell surface, conidia adhesion, and biofilm formation, features usually associated with the CWI pathway. Germlings of $\Delta pphA$ showed similar adherence to polystyrene and fibronectin-coated plates compared to the wild type. However, adhesion assay with conidia displayed significant reduction in adherence. Again, these results show that conidia and hyphae may exhibit differences in surface carbohydrates that result in alterations in important mechanisms during the host infection process, including adhesion properties. Immunoblot analysis showed that MpkA and SakA phosphorylation in response to CR and osmotic stresses are not affected by PphA. Moreover, our results demonstrated that $\Delta pphA$ and wild-type showed similar growth in presence of protein kinase C inhibitors. The activation of the CWI in fungi is dependent of the protein kinase C-mediated mitogen-activated protein kinase (PKC1-MAPK) pathway (Monge et al., 2006; Levin, 2005). Thus, more studies are required to understand which signaling pathways are being regulated by this phosphatase under cell wall stresses.

In this work, we demonstrated that PphA contributes to virulence of *A. fumigatus*. Comparative analysis of wild-type and the mutant strain in a neutropenic murine model of pulmonary aspergillosis showed that the $\Delta pphA$ is avirulent. The reduction of GAG and increased β -(1,3)-glucan in the $\Delta pphA$ cell wall can contribute to recognition of this strain by the host immune system and to induction of inflammatory responses. Corroborating this hypothesis, we observed that the cytokine Tumour

Necrosis Factor alpha (TNF- α), released from macrophages in response to *A. fumigatus* exposure (Taramelli et al., 1996; Hayashi et al., 2005), was increased in presence of the *ApphA* conidia.

In summary, this study shows that the loss of specific MAP kinases and phosphatases had striking effects on the cell surface, adhesion of the conidia and germings, and biofilm formation in *A. fumigatus*. However, how the upstream sensors trigger the MAP kinase cascades and which transcriptional regulators are being activated during the biofilm formation need to be elucidated.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at <http://dx.doi.org/10.1016/j.tcs.2018.03.002>.

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Durante o período de desenvolvimento da tese, além dos artigos nela mencionados, foram concluídos e realizados outros trabalhos em paralelo:

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2020 - Jacob L. Steenwyk, Abigail L. Lind, Laure N.A. Ries, Thaila F. dos Reis, **Lilian P. Silva**, Fausto Almeida, Rafael W. Bastos, Thais Fernanda de Campos Fraga da Silva, Vania L.D. Bonato, Andre Moreira Pessoni, Fernando Rodrigues, Huzefa A. Raja, Sonja L. Knowles, Nicholas H. Oberlies, Katrien Lagrou, Gustavo H. Goldman and Antonis Rokas. "Pathogenic Allodiploid Hybrids of *Aspergillus* Fungi". *Current Biology*, v.30, p.1–13.

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2020 - Rafael Bastos, Clara Valero, **Lilian Silva**, Taylor Schoen, Milton Drott, Veronica Brauer, Rafael Silva-Rocha, Abigail Lind, Jacob Steenwyk, Antonis Rokas, Fernando Rodrigues, Agustin Resendiz-Sharpe, Katrien Lagrou, Marina Marcet-Houben, Toni Gabaldón, Erin McDonnell, Ian Reid, Adrian Tsang, Berl Oakley, Flávio Loures, Fausto Almeida, Anna Huttenlocher, Nancy Keller, Laure Ries, and Gustavo Goldman. "Functional characterization of clinical isolates of

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Rodrigues, Iran Malavazi and Gustavo H. Goldman. The Influence of Genetic Stability on *Aspergillus fumigatus* Virulence and Azole Resistance. G3-Genes Genomes Genetics, v. 8, p. g3.300265.