UNIVERSIDADE FEDERAL DO RIO GRANDE DO SUL

CENTRO DE BIOTECNOLOGIA

PROGRAMA DE PÓS-GRADUAÇÃO EM BIOLOGIA CELULAR E MOLECULAR

AVALIAÇÃO DO PAPEL DE PROTEÍNAS Hsp12 NA VIRULÊNCIA E RESPOSTA AO ESTRESSE DE *Cryptococcus gattii*

Dissertação de Mestrado

Heryk Motta de Souza

Porto Alegre, Outubro de 2019

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Dissertação submetida ao Programa de Pós-Graduação em Biologia Celular e Molecular do Centro de Biotecnologia da Universidade Federal do Rio Grande do Sul como requisito parcial para obtenção do Grau de Mestre.

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SUMÁRIO

LISTA DE ABREVIATURAS, SÍMBOLOS E UNIDADES

%	Por cento
°C	Graus Celsius
µg	Micrograma
µL	Microlitro
µM	Micromolar
AIDS	Síndrome da imunodeficiência adquirida (do inglês *Acquired immunodeficiency syndrome*)
cDNA	Ácido desoxirribonucleico complementar
DNA	Ácido desoxirribonucleico
DNase	Desoxirribonuclease
dNTP	Desoxirribonucleotídeo trifosfatado
g	Grama
h	Hora
HIV	Vírus da *imunodeficiência humana (*do inglês Human immunodeficiency virus)
Hsp	Proteina de choque térmico (do inglês *Heat shock protein*)
Kg	Quilograma
mg	Miligrama
MIC	Concentração inibitória mínima (do inglês *Minimum inhibitory concentration*)
min	Minuto
mL	Mililitro
mM	Milimolar
mRNA	Ácido ribonucleico mensageiro
nM	Nanomolar
OD	Densidade ótica (do inglês *optical density*)
PAMP	Padrões moleculares associados ao patógeno (do inglês *Pathogen-associated molecular patterns*)
PBS	Tampão fosfato-salino (do inglês *Phosphate buffered saline*)
PCR	Reação em cadeia da polimerase (do inglês *Polymerase chain reaction)*

pmol	Picomol
PRR	Receptores de reconhecimento de padrão (do inglês *Pattern recognition receptors*)
qPCR	Reação em cadeia da polimerase quantitativo em tempo real (do inglês *quantitative Polymerase chain reaction)*
RNA	Ácido ribonucleico
s	Segundo
sHsp	Pequena proteína de choque térmico (do inglês *Small heat shock protein*)

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RESUMO

O estabelecimento de infecções de leveduras patogênicas do gênero *Cryptococcus* depende da expressão de características que permitem a sua adaptação ao ambiente do hospedeiro. Os principais determinantes de patogenicidade são: a sua capacidade de se desenvolver na temperatura do hospedeiro (37 °C) e a produção de melanina na superfície celular e de capsula polissacarídica. Outros determinantes são importantes para o estabelecimento da infecção, entre eles, a capacidade de suplantar a resposta de estresse desenvolvida pelas defesas do hospedeiro. Para tal, ocorrem alterações importantes no perfil de expressão gênica na levedura. Foi demonstrado que as proteínas de choque térmico tem atividade fundamental na viabilidade das leveduras no ambiente do hospedeiro, pois modulam determinantes de patogenicidade e a própria resposta imunológica do hospedeiro. Neste contexto, caracterizamos duas proteínas Hsp12 (genes parálogos *HSP12.1* e *HSP12.2*) de choque térmico de *C. gattii*. Mostramos que a expressão do gene *HSP12.1* está aumentada durante o choque térmico e que a sua inativação, por construção de mutantes, resultou em fenótipo de sensibilidade ao composto FK506, um inibidor da via da calcineurina. Esta via é fundamental para a resposta e adaptação da levedura a diferentes tipos de estresse celular. O mutante de *HSP12.1* é mais sensível ao estressor H_2O_2 e ocorre acumulo intracelular de ROS. Estes mutantes mostram também um fenótipo de sensibilidade ao SDS que é um estressor de membrana plasmática, sugerindo que a proteína *HSP12.1* é provavelmente uma chaperona na membrana plasmática. Mostramos também que a inativação do outro gene *HSP12.2* não resulta em alterações fenotípicas detectáveis, indicando que este parálogo ou é inativo na levedura selvagem ou

que a sua inativação provoca algum efeito compensatório. O resultado mais importante, do ponto de vista da relação *C. gattii* com o hospedeiro é que a inativação de *HSP12.1* resulta na redução da taxa de fagocitose por macrófagos murinos que resulta em hipovirulência do mutante em modelo de infecção sistêmica em modelo invertebrado. Assim, os resultados contribuem para o entendimento das relações *C. gattii* com seus hospedeiros e mostram a importância do gene *HSP12.1* para a sua adaptação ao estresse durante a infecção.

ABSTRACT

The establishment of pathogenic yeast infections of the genus *Cryptococcus* relies on the expression of determinants of pathogenicity that allow its adaptation to the host environment. The main determinants of pathogenicity are its ability to grow at host temperature (37 °C), the production of melanin on the cell surface, and a polysaccharide capsule. Other determinants are important for the establishment of the infection, including the ability to overcome the stress response developed by the host's defenses. For this purpose, significant changes occur in the gene expression profile in yeast. Heat shock proteins (HSP) have been shown to play a fundamental role in the viability of yeasts in the host environment as they modulate determinants of pathogenicity and the host's immune response. In this context, we characterized two Hsp12 proteins (genes *HSP12.1* and *HSP12.2*) in *C. gattii*. We demonstrated that *HSP12.1* gene expression is increased during heat shock, and its inactivation, through the construction of a null mutant, resulted in a sensitivity phenotype to the compound FK506, an inhibitor of the calcineurin pathway. This pathway is essential for the response and adaptation of yeast to different types of cellular stress. The HSP12.1 mutant also showed increased sensitivity to the H_2O_2 stressor and intracellular accumulation of ROS. These mutants also showed an SDS-sensitive phenotype, which acts by destabilizing the plasma membrane, suggesting that Hsp12.1 acts as a chaperone in the plasma membrane. We also demonstrated that the inactivation of the other *HSP12.2* gene does not result in detectable phenotypic changes, indicating that this paralog is either inactive in wild *C. gattii* or that its inactivation causes some compensatory effect. The most important result, from the point of view of the *C. gattii* interaction with the host, is that the

inactivation of *HSP12.1* leads to a reduction in the rate of phagocytosis by murine macrophages associated with a hypovirulence phenotype in a systemic infection model in an invertebrate model. Thus, these results contribute to the understanding of *C. gattii*'s relations with its hosts and demonstrate the importance of the HSP12.1 gene for its adaptation to stress during infection.

1. Introdução

Cryptococcus gattii é uma levedura basidiomicética e um dos agentes causais da cryptococose humana, juntamente com *Cryptococcus neoformans*. Essa infecção fúngica invasiva é adquirida pela inalação de esporos ou mesmo células dessecadas encontradas no ambiente. A progressão da doença se dá pela colonização do tecido pulmonar, podendo ocorrer evasão das defesas do hospedeiro, disseminação pelo sistema circulatório e, na forma mais grave, levar a um quadro de meningite fúngica (Figura 1).

Estima-se que anualmente cerca de 181.000 indivíduos vão a óbito devido à meningite criptocócica em nível mundial (Rajasingham *et al*., 2017). No Brasil, a criptococose lidera, entre demais micoses sistêmicas, em número de óbitos. É fundamental enfatizar, entretanto, que dados epidemiológicos encontram-se subestimados devido à não notificação aos centros de vigilância ou mesmo a falta de diagnóstico de *causa mortis* (Alves Soares *et al*., 2019).

1.1. *Cryptococcus neoformans* e *Cryptococcus gattii*

C. neoformans e *C. gattii* divergiram de um ancestral comum, sendo que as espécies se diferenciam por sua epidemiologia, características sorológicas e moleculares (Lin & Heitman, 2006). *C. neoformans* é predominantemente associado a quadros de infecção em indivíduos imunocomprometidos ou imunossuprimidos. Neste contexto, destacam-se indivíduos portadores do vírus HIV, transplantados e portadores de doenças malignas. *C. neoformans* pode ser facilmente isolado de excretas de pombos, sendo este seu principal reservatório urbano (Maziarz & Perfect, 2016).

C. gattii, por sua vez, pode acometer tanto indivíduos imunocomprometidos quanto imunocompetentes. Acreditava-se que *C. gattii* possuía distribuição limitada à região tropical e subtropical (Kwon-chung & Bennett, 1984), porém, um surto em Vancouver, Canadá mostrou que este patógeno também possui ampla distribuição em regiões de clima temperado (Kidd *et al*., 2004). *C. gattii* é frequentemente associado a cascas de árvores em decomposição, em especial destacam-se árvores pertencentes ao gênero *Eucalyptus* (Ellis & Pfeiffer, 1990).

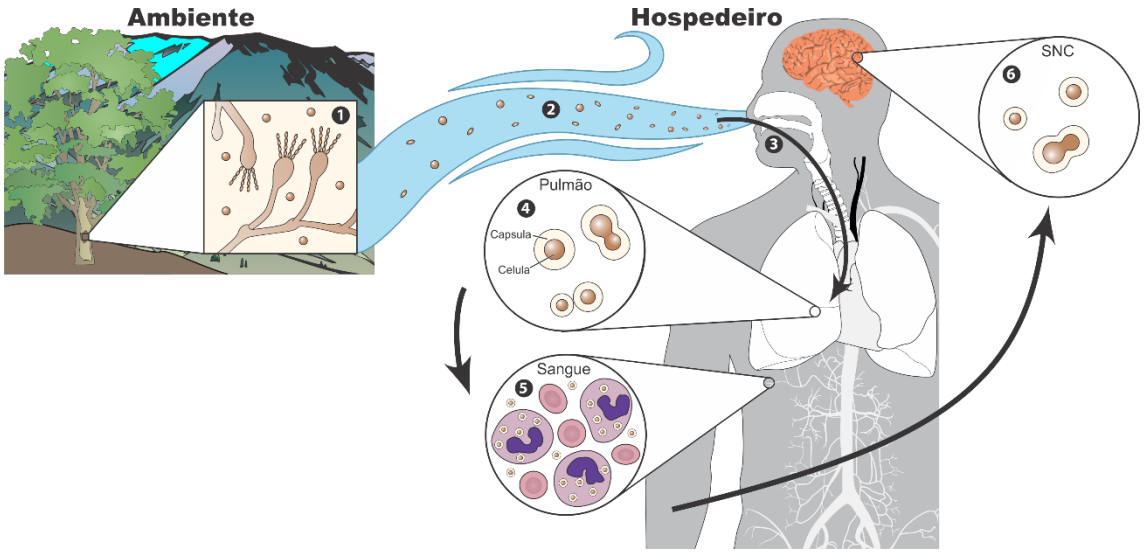

Figura 1. Ciclo de infecção de *Cryptococcus gattii*. Células dessecadas ou esporos da levedura são encontrados no ambiente (1). A infecção ocorre através da inalação destas células ou esporos (2) que acabam se depositando no pulmão do hospedeiro (3). Ocorre a proliferação no tecido pulmonar com produção de uma cápsula polissacarídica protetora (4). Após colonização do tecido pulmonar, células podem se disseminar pelo sistema circulatório (5) onde, em sua forma mais grave, ocorre colonização do sistema nervoso central (6). Adaptado de Centers for Disease Control and Prevention, 2019. (https://www.cdc.gov/fungal/diseases/cryptococcosis-gattii/causes.html).

1.2. O primeiro contato com o hospedeiro

Como descrito previamente, a infecção por *Cryptococcus* sp. se inicia após inalação de células ou esporos dessecados seguida da colonização do tecido pulmonar. Desta forma, é fundamental uma compreensão do ambiente pulmonar do hospedeiro para entendermos o processo de infecção. O sistema respiratório humano possui uma complexa e especializada resposta imunológica devido a frequente exposição a particulados e microrganismos. Diversas barreiras justificam o limitado número de microrganismos capazes de infectar o trato respiratório inferior em condições normais (Esher, Zaragoza, & Alspaugh, 2018).

Dentre os limitantes prévios à resposta imunológica podemos destacar: a temperatura do hospedeiro (37 °C), representando uma primeira dificuldade para patógenos; a escassa quantia de nutrientes no ambiente pulmonar em condições normais (Dickson, Erb-Downward, Martines, & Huffnagle, 2016); e a presença de surfactantes pulmonares compostos de proteínas com atividade antimicrobiana e profagocíticas (Glasser & Mallampalli, 2012).

A primeira linha de defesa imunológica encontrada no hospedeiro são os macrófagos alveolares (Erwig & Gow, 2016). Para exercerem sua atividade, essas células fagocíticas necessitam reconhecer corpos estranhos para então fagocitá-los. Esse reconhecimento pode ser realizado através de padrões moleculares associados ao patógeno (PAMPs) por receptores de reconhecimento de padrão (PRR) ou através do reconhecimento de corpos estranhos/patógenos opsonizados por proteínas como as que compõe o surfactante pulmonar (Campuzano & Wormley, 2018). Após o processo de

fagocitose, o fagossomo é fundido ao lisossomo formando o fagolisossomo. O fagolisossomo é caracterizado por um pH ácido, presença de enzimas hidrolíticas e uma elevada concentração de radicais livres (García-Rodas & Zaragoza, 2012; Mansour, Reedy, Tam, & Vyas, 2014), sendo considerado um ambiente hostil para patógenos.

1.3. Determinantes de virulência

Para o sucesso da infecção, *C. neoformans* e *C. gattii* expressam determinantes de virulência que auxiliam na evasão das barreiras do hospedeiro supracitadas. Dentre eles, destaca-se a termotolerância, produção de melanina e produção de uma cápsula polissacarídica. O papel desses determinantes de virulência é fundamental para infecção e serão discutidos em detalhes a seguir.

1.3.1. Termotolerância

A capacidade de adaptação à temperatura do hospedeiro é essencial para o processo de infecção. Sendo assim, vias de sinalização que governam a termotolerância tornaram-se alvos extensivos de estudo (Yang *et al*., 2017).

Dentre as vias de sinalização responsáveis pela termotolerância, é notável a importância da via da calcineurina. Esta via de sinalização é ativada pela detecção de alterações transitórias nas concentrações plasmáticas de cálcio (Ca^{2+}). Ao serem ativados, sensores de estresses diversos promovem um influxo de cálcio para o citoplasma da levedura. Esse aumento na concentração de cálcio é detectado pela proteína calmodulina que, por sua vez, ativa a fosfatase calcineurina. A calcineurina ativada orquestra uma ramificada via de sinalização onde atuará em diferentes alvos, como na desfosforilação do fator de transcrição Crz1. Este, quando translocado para o núcleo, promove a

expressão de genes relacionados ao metabolismo e resposta ao estresse (Chow *et al*., 2017; Cyert, 2003; Park *et al*., 2016).

Em concordância com estes dados, a inativação da subunidade catalítica da calcineurina, tanto em *C. gattii* quanto em *C. neoformans,* mostraram-se capazes de levar um defeito no crescimento a 37 °C além de serem imprescindíveis para virulência das leveduras (Chen, Lehman, Lewit, Averette, & Heitman, 2013; Odom *et al*., 1997).

1.3.2. Melanina

Melanina é um pigmento de coloração escura, carregado negativamente e insolúvel em meio aquoso e solventes orgânicos. Sua produção é mediada pela enzima lacase que promove a oxidação de catecolaminas (Casadevall, Rosas, & Nosanchuk, 2000). A melanina é importante durante o ciclo de vida de *Cryptococcus* sp. no meio ambiente. Sua atuação não se limita apenas à proteção contra radiação ultra violeta e ionizante, uma vez que possui um importante papel na defesa contra predadores como amebas (Dadachova *et al*., 2007; Yulin Wang, Aisen, & Casadevall, 1995).

Protegido da radiação ultravioleta no interior do hospedeiro, *Cryptococcus* sp. passa a se beneficiar do efeito antioxidante da melanina. Células melanizadas são mais resistentes a espécies reativas de oxigênio e nitrogênio produzidas por células fagocíticas (Casadevall *et al*., 2000; Steenbergen, Shuman, & Casadevall, 2001; Y. Wang & Casadevall, 1994; Yulin Wang *et al*., 1995).

Além da atuação da melanina, a própria atividade da lacase tem importante papel na proteção contra estresse oxidativo. A lacase possui,

também, atividade de ferro oxidase que leva a oxidação de Fe (II) à Fe (III). Fe (III) por sua vez é reduzido e utilizado no balanço de espécies redutoras e oxidativas nas células de defesa do hospedeiro, sendo convertido à Fe (II) e novamente à Fe (III). Dessa forma, a lacase atua de forma protetora, juntamente com a melanina, durante situações de estresse redox e sua deleção leva a perda total da virulência de *Cryptococcus* sp. (Liu, Tewari, & Williamson, 1999; Zhu & Williamson, 2004).

1.3.3. Cápsula polissacarídica

A produção de uma cápsula polissacarídica é o fator de virulência mais notável de *Cryptococcus* sp. (Figura 2). Sua composição predominante é o polissacarídeo polimérico glucuronoxilomanana (GXM), correspondendo a 90% da massa capsular, seguido do polissacarídeo galactoxilomanana (GalXM) e manoproteínas em ínfima quantidade. Devido à sua grande massa molecular, GXM é sintetizado no interior da célula fúngica e posteriormente secretado para formação da cápsula na superfície celular (Agustinho, Miller, Li, & Doering, 2018; McFadden, De Jesus, & Casadevall, 2006; McFadden, Fries, Wang, & Casadevall, 2007).

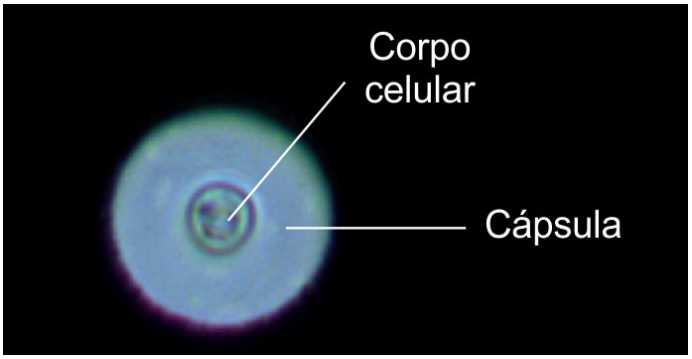

Figura 2. Cápsula polissacarídica de *C. gattii*. Microscopia óptica de células de *C. gattii* na presença de tinta nanquim.

O processo de indução da cápsula polissacarídica é extremamente complexo e fenótipos variados são observados de forma estimulo-dependente (O'Meara & Alspaugh, 2012). Uma vez formada, seu papel é fundamental na patogênese de *Cryptococcus* considerando que mutantes desprovidos não foram capazes de causar infecção em modelo murino (Fromtling, Shadomy, & Jacobson, 1982).

A cápsula polissacarídica de *Cryptococcus* sp. possui papel diversificado durante a infecção. Atuando como um escudo e mascarando PAMPs, a cápsula polissacarídica reveste a célula, evitando a fagocitose (Erwig & Gow, 2016). A cápsula polissacarídica e o próprio GXM impedem a ligação de componentes da resposta imune inata presentes no surfactante pulmonar, evitando agregação e opsonização das células fúngicas (Giles, Zaas, Reidy, Perfect, & Wright, 2007; Van De Wetering, Coenjaerts, Vaandrager, Van Golde, & Batenburg, 2004). Além de sua função no impedimento do reconhecimento da célula fúngica por fagócitos do hospedeiro, GXM possui um importante caráter modulatório da resposta imunológica mediada por citocinas (Monari *et al*., 2005; Vecchiarelli, 2000). Por outro lado, o material capsular é fundamental para a replicação da levedura dentro dos fagócitos, caracterizando *Cryptococcus* sp. como um patógeno intracelular facultativo (Feldmesser, Kress, Novikoff, & Casadevall, 2000).

A capacidade de *Cryptococcus* sp. de parasitar macrófagos, mediada pela produção da cápsula polissacarídica em conjunto com demais fatores de virulência descritos, é de grande importância para colonização do sistema nervoso central do hospedeiro. Uma vez dentro de macrófagos, *Cryptococcus*

sp. pode utilizá-los como veículo para atravessar a barreira hematoencefálica (Esher *et al.*, 2018; J. W. Kronstad *et al*., 2011; O'Meara & Alspaugh, 2012).

1.4. Homeostase do metabolismo primário

Como descrito anteriormente, os fatores de virulência têm papel central na adaptação e manutenção da viabilidade das células fúngicas durante a infecção. Porém, modificações em seu metabolismo e em diversas funções são necessárias para atingir o sucesso da infecção.

Em uma análise dos transcritos de *C. neoformans* durante a fase inicial da infecção no ambiente pulmonar do hospedeiro Hu e colaboradores demonstraram a grande necessidade de adaptação da levedura para causar a infecção. Aumento na transcrição de genes de metabolismo alternativo de carbono demonstram o hospedeiro como um ambiente nutricionalmente pobre em glicose. Ainda, genes cujos produtos estão envolvidos na adaptação ao estresse aparecem induzidos, como a lacase e superóxido dismutase, responsáveis pela resposta a espécies reativas (Hu, Cheng, Sham, Perfect, & Kronstad, 2008).

Um perfil similar ao encontrado no ambiente pulmonar foi descrito em células internalizadas por fagócitos. *C. neoformans* também demonstrou profundas alterações na expressão de genes relacionados a transportadores em geral e vias metabólicas, sugerindo o fagolisossomo como um ambiente nutricionalmente pobre. A transcrição aumentada de genes de resposta ao estresse e cascatas de sinalização também demonstram o grande estresse ao qual as células fúngicas encontram-se submetidas (Fan, Kraus, Boily, & Heitman, 2005; J. Kronstad *et al*., 2012).

1.5. Proteínas de choque térmico (HSP)

Dentre as alterações metabólicas e enzimáticas que atuam na manutenção da homeostase celular, podemos destacar as proteínas de choque térmico (Hsp). Hsps podem ser encontradas em praticamente todos compartimentos celulares (Kregel, 2002). Hsps são usualmente classificadas em, ao menos, 6 famílias de acordo com sua massa molecular: Hsp40, Hsp60, Hsp70, Hsp90, Hsp100 e pequenas Hsps (sHsp) (Hartl, Bracher, & Hayer-Hartl, 2011).

Embora amplamente associadas unicamente com a função característica de chaperonas, Hsps apresentam funções diversificadas. Processos como ubiquitinação, estabilização da membrana plasmática, composição do citoesqueleto e regulação de cascatas de sinalização são alguns exemplos de funções exercidas por Hsps em células fúngicas (Tiwari, Thakur, & Shankar, 2015).

1.5.1. Hsp40

São caracterizadas pela presença de um domínio J contendo 70 aminoácidos localizado de forma randômica na extensão da proteína. Esse domínio é responsável por regular a atividade hidrolítica de ATP de proteínas da família Hsp70. Hsp40 pode ligar-se ao substrato proteico e, através de seu domínio J, promover a hidrólise de ATP por Hsp70 e transferir o polipeptídio para Hsp70 (Qiu, Shao, Miao, & Wang, 2006).

1.5.2. Hsp60

Essencial durante o crescimento a elevadas temperaturas, a Hsp60 atua no dobramento de proteínas na matriz mitocondrial. Dependendo de qual

substrato será auxiliado no redobramento, Hsp10 se faz necessária de forma auxiliar. Ainda, a superexpressão de Hsp60 mostrou um efeito protetor durante o estresse oxidativo na presença de menadiona e peróxido de hidrogênio, demonstrando a versatilidade de contextos em que a função de Hsp60 tem efeito protetivo (Burnie, Carter, Hodgetts, & Matthews, 2006).

1.5.3. Hsp70

Como já descrito, Hsp70 possui atividade hidrolítica de ATP e em associação ou mesmo na ausência de Hsp40, atua na estabilização de regiões hidrofóbicas de segmentos de polipeptídios. Membros da família também possuem atividade na regulação de resposta ao choque térmico e translocação de proteínas através de membranas (Bukau & Horwich, 1998; Burnie *et al.*, 2006).

1.5.4. Hsp90

Com principal papel no dobramento de proteínas, com e sem o gasto de ATP, Hsp90 possui diversas outras funções e sua ausência afeta a virulência de fungos patogênicos. Hsp90 possui importante papel na tolerância e desenvolvimento de resistência a antifungos através da modulação da composição de biofilmes em *Candida albicans* e *Aspergillus fumigatus* (Lamoth, Juvvadi, & Steinbach, 2016; Robbins *et al.*, 2011; Xie, Polvi, Shekhar-Guturja, & Cowen, 2014).

1.5.5. Hsp100

Ao contrário da Hsp70 e Hsp40, membros da família Hsp100 não evitam a agregação de proteínas, porém, sua atividade central envolve reativação de proteínas que foram desnaturadas (Burnie *et al.*, 2006).

1.5.6. sHsp

Subdivididas de forma análoga às proteínas de choque térmico, sHsp também são agrupadas por sua massa molecular. Ao contrário das proteínas de choque térmico, sHsps são menos conservadas entre diferentes reinos (Haslbeck & Vierling, 2015). Entre os diversificados indivíduos que fazem parte deste grupo, Hsp12 destaca-se por sua distinta atividade na estabilização da membrana plasmática de fungos em condições de estresse osmótico e térmico (Burnie *et al.*, 2006; Tiwari *et al.*, 2015; Verghese, Abrams, Wang, & Morano, 2012).

1.5.6.1. Hsp12

Indetectável no crescimento exponencial, Hsp12 apresenta elevada expressão durante a fase estacionária de *S. cerevisiae* (Praekelt & Meacock, 1990). Em sua forma solúvel, encontra-se amplamente dispersa no citoplasma, porém, quando associada à membrana plasmática, assume sua estrutura terciária. Sua principal função é a manutenção da membrana plasmática, regulando sua fluidez e integridade em condições de estresse (Sales, Brandt, Rumbak, & Lindsey, 2000; Welker *et al.*, 2010).

Na levedura patogênica *C. albicans*, Hsp12 possui sua expressão regulada por estressores osmóticos, durante estresse oxidativo e na presença de CO_2, além de ser induzida pela molécula de *quorum sensing*, farnesol. Por atuar na membrana plasmática, sua inativação aumenta a sensibilidade aos antifúngicos da classe dos azoles e polienos (Fu, de Sordi, & Mühlschlegel, 2012; Sheth, Mogensen, Fu, Blomfield, & Mühlschlegel, 2008).

1.6. Hsps em *Cryptococcus* sp.

São limitados os dados encontrados sobre a caracterização de Hsps em *C. neoformans* e *C. gattii*. Hsp70 e Hsp90 são os dois grupos que apresentam maior detalhamento considerando sua ligação com a virulência demonstrada em outros fungos patogênicos.

1.6.1. Hsp70

Associada à cápsula de *C. neoformans*, Hsp70 é reconhecida por soro de camundongos infectados pela levedura e, como demonstrado em dois diferentes estudos, pelo soro de pacientes com criptococose (Kakeya *et al.*, 1997, 1999; Silveira *et al.*, 2013).

Membros da família Hsp70 em *C. neoformans* também tiveram sua função caracterizada, onde foi demonstrado que a Hsp70 possui capacidade imunomodulatória na fase inicial da infecção. Há favorecimento da replicação fúngica no sítio de infecção através da redução da produção de óxido nítrico por células do hospedeiro (Eastman *et al.*, 2015; Silveira *et al.*, 2013).

1.6.2. Hsp90

Em *Cryptococcus* sp., assim como em *C. albicans* e *A. fumigatus*, Hsp90 também é capaz de causar impacto durante a infecção. Em *C. neoformans*, Hsp90 possui envolvimento com termotolerância, indução e manutenção da cápsula polissacarídica e resistência à equinocandinas a 37 °C (Chatterjee & Tatu, 2017). A inibição de Hsp90, por sua vez, demonstrou promissores resultados na inibição da formação de biofilme quando associado às principais classes de antifúngicos, além de potencializar seu efeito. Além disso, em associação com a administração de fluconazol, a inibição farmacológica de Hsp90 demonstrou considerável aumento na taxa de sobrevivência de

Caenorhabditis elegans infectados por *C. neoformans* e *C. gattii* (Cordeiro *et al.*, 2016).

Membros de ambas famílias Hsp70 e Hsp90, também foram descritos como componentes de vesículas extracelulares de *C. neoformans,* importantes carregadores de determinantes de virulência e conhecidos imunomoduladores (Rodrigues *et al.*, 2008).

1.6.3. Hsp12

Hsp12 também foi parcialmente caracterizada em *C. neoformans*, onde duas cópias deste gene podem ser encontradas, e sua expressão é regulada pelas vias clássicas de sinalização de estresse *High-osmolarity glycerol* (HOG) e AMP cíclico (cAMP). Com a construção de mutantes simples e duplo para estes genes, denominados *HSP12* e *HSP122*, foi demonstrado seu envolvimento na resistência a metais pesados e seu papel redundante na resistência à anfotericina B (Maeng *et al.*, 2010).

Como citado anteriormente, Hsps são fundamentais na resposta ao estresse, como no ambiente do hospedeiro. Também, Hsps já foram caracterizadas como moduladores de determinantes de virulência em fungos patogênicos (Burnie *et al.*, 2006). Dessa forma, associado com a falta de informações sobre Hsps e sHsps em *C. gattii*, e seu papel na patobiologia causada por este fungo, o presente trabalho tem por objetivo a caracterização de suas Hsp12. Para a caracterização funcional das duas Hsp12 codificadas pelos genes *HSP12.1* (CNBG_2441) e *HSP12.2* (CNBG_5853), realizou-se a caracterização fenotípica de linhagens deletadas para os genes citados.

2. Objetivos

2.1. Objetivo geral

Caracterizar a função das Hsp12 (codificadas pelos genes *HSP12.1* e *HSP12.2*) de *C. gattii* na resposta ao estresse e seu impacto na virulência.

2.2. Objetivos específicos

2.2.1. Identificação de genes codificadores de proteínas contendo o domínio de Hsp9/12 no genoma de *C. gattii*;

2.2.2. Determinação do perfil de expressão do gene *HSP12.1* durante o choque-térmico;

2.2.3. Construção de linhagens nulas e complementadas para os genes *HSP12.1* e *HSP12.2* de *C. gattii*;

2.2.4. Análises fenotípicas em condições de estresse das linhagens mutantes;

2.2.5. Ensaios de interação patógeno-hospedeiro das linhagens construídas com macrófagos murinos e em modelo invertebrado.

3. Manuscrito

“The Hsp12.1 has a major role in stress response and virulence of *Cryptococcus gattii*”

Manuscrito será submetido à revista mSphere.

“The Hsp12.1 has a major role in stress response and virulence of *Cryptococcus gattii*”

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ABSTRACT

Cryptococcus gattii is one of the etiological agents of cryptococcosis, a disease responsible for more than 180.000 deaths each year. In order to achieve a successful infection, *C. gattii* have to adapt to the harsh environment found in the host tissues, including a highly specialized immune system and a poor nutritional condition. To circumvent such non-favorable conditions, *C. gattii* cells employ a diversified set of tools to keep their proper homeostasis. The adaptation include the expression of a remarkable and diversified group of heat shock proteins. One class of these proteins are the Hsp12, whose roles in fungal virulence is poorly documented. In this study, *C. gattii* Hsp12.1 and Hsp12.2 had their function characterized employing the generation of null mutants and complemented strains. *hsp12.1Δ* mutant cells were more sensitive to membrane and oxidative stressors and also displayed intracellular ROS accumulation. In addition, *hsp12.2Δ* null strain did not displayed detectable phenotype changes, suggesting a compensatory effect by Hsp12.1. Also, *HSP12.1* disruption altered the sensibility to phagocytosis by macrophages and caused a reduction on *C. gattii* virulence. This data suggest that Hsp12.1 has an important role in plasma membrane stabilization and it's important for *C. gattii* virulence and host adaptation.

IMPORTANCE

Cryptococcus gattii cells have a set of tools to maintain its proper homeostasis during their distinct lifestyles, including infection. Among these, heat shock proteins are a large group of diversified, poorly understood, and stress response components. This work showed the importance to virulence and membrane stabilization of one Hsp12

of *C. gattii*. This data contributes to a better understanding of this complex pathogen and may elect possible new targets to future therapeutics approach.

KEYWORDS

Cryptococcus gattii, Hsp12, Heat shock protein, Plasma membrane

INTRODUCTION

Cryptococcus neoformans and *Cryptococcus gattii* are the etiological agents of cryptococcosis, a disease responsible for 181,100 deaths worldwide (1). *C. neoformans* mainly affects immunocompromised hosts, being mostly associated with HIV-infected patients. In contrast, *C. gattii* infections predominate in immunocompetent hosts (2, 3). The infection generally begins with the inhalation of basidiospores or dissected yeast cells. After colonizing the lungs, fungal cells can disseminate through the bloodstream to the CNS, leading to meningitis (4).

In order to survive and replicate during infection, cryptococcal cells rely on the production of several virulence determinants to deal with a hostile environment and a highly specialized immune system, including a polysaccharide capsule, extracellular enzymes secretion, biofilm formation, melanin production and thermotolerance (5, 6). Whilst the mechanisms played by such virulence determinants allow cryptococcal survival during infection, basic aspects of cell homeostasis are also required for the yeast cells survival (7). Being part of a wide range of biological process like ubiquitination, membrane stabilization, and protein folding, heat shock proteins (Hsp) are remarkable components in stress response (8). Hsps are usually categorized into at least 6 major distinct families according to their molecular masses: Hsp40, Hsp60, Hsp70, Hsp90, Hsp100 and the small HSPs (9).

Some cryptococcal proteins from the Hsp70 family, one of the most studied and characterized (10), were already described to be recognized by serum of infected mice. In addition, such proteins are capable to modulate the host immune

response, highlighting the importance of these proteins during the infection process (11–13). Cryptococcal Hsp90 also displays association with virulence, as it regulates capsule formation and alter the sensitivity to echinocandins (14). Pharmacological inhibition of Hsp90 led to an alteration of capsule size and virulence attenuation in *Caenorhabditis elegans* infection model, demonstrating that these proteins can be used as target for drug development (15).

Compared to well-studied fungal Hsp70 and Hsp90 families, little is known about the class of small Hsps. In *S. cerevisiae,* Hsp12 stabilizes the plasma membrane like a lipid chaperone (16). However, the role of Hsp12 orthologs in pathogenic fungi are only speculated (17, 18). In this study, we demonstrate that the inactivation of one (Hsp12.1) of the two paralogs identified in *C. gattii* leads to higher sensitivity to oxidative and membrane stressors, as well as impaired phagocytosis by J774.16 macrophage and hypovirulence, as evaluated in a non-mammalian host. The same phenotypes could not be observed when the other paralog (Hsp12.2) was inactivated, suggesting functional divergence between these two proteins.

RESULTS

HSP12 identification and expression analysis

In order to characterize proteins from the small Hsp family in *C. gattii* R265, we performed a search for proteins containing the Heat shock protein 9/12 PFam domain (PF04119) using the FungiDB platform (19). Two predicted genes, named *HSP12.1* (CNBG_2441) and *HSP12.2* (CNBG_5853), were found and code for Hsps with predicted molecular masses of 8.8 and 7.7 kDa, respectively.

As *HSP* genes present a conserved response to heat shock stress (8), we evaluated the transcript levels of *HSP12.1* in a time course fashion after a temperature shift from 30 °C to 39 °C. A drastic increased expression could be noted after 40 minutes of heat-shock (Fig 1), resembling its orthologs from *S. cerevisiae* and *C. albicans* (17, 20).

Cryptococcal cells lacking *HSP12.1* displayed hypersensitivity to calcineurin pharmacological inhibition

In order to access whether Hsp12.1 and Hsp12.2 could mediate the response to stress in *C. gattii*, single and double null mutant strains were constructed by replacing the *HSP* genes with a selection marker employing biolistics. Since the calcineurin signaling pathway is pivotal in stress response and growth at higher temperatures, we evaluated whether the inhibition of this pathway using FK506 would impact the fitness of cells lacking *HSP12.1*. While WT cells displayed a minimum inhibitory concentration (MIC) higher than 2 µg/mL, FK506 at 0.25 µg/mL was sufficient to inhibit completely the growth of *hsp12.1Δ* strain (Fig 2A). Despite

the hypersensitivity to calcineurin inhibition observed in cells lacking *HSP12.1*, no differences could be found among the growth rate of WT and *hsp12.1Δ* strains at 37 °C (Fig 2B). In addition, the absence of *HSP12.1* or *HSP12.2* did not alter the survival rate after heat shock for distinct periods (10, 20, and 40 min) at 39 °C (Fig 2C), suggesting that *HSP12.1* and *HSP12.2* could be involved in the response to other types of stresses.

HSP12.1 absence leads to reactive oxygen species (ROS) accumulation and low tolerance to oxidative stress

As the calcineurin pathway also regulates the responses to oxidative stress, we sought to determine if Hsp12.1 and Hsp12.2 would be involved in this adaptation. Sensitivity to oxidative stress was evaluated by spotting plate assay in YNB medium added of hydrogen peroxide. The null mutant *hsp12.1Δ* and the double mutant *hsp12.1Δ/hsp12.2Δ* displayed a consistent reduced growth rate compared to WT, a phenotype not observed for *hsp12.2Δ* mutant strain (Fig 3A). The reintroduction of the gene sequence into the mutant strain reconstitutes the phenotype to those observed in WT cells. To further investigate the hypersensitivity of *hsp12.1Δ* to hydrogen peroxide, the intracellular ROS levels were determined by flow cytometry employing the probe dichlorofluorescin diacetate. After exposure of WT, *hsp12.*1Δ and *hsp12.1Δ*::*HSP12.1* cells at 37 °C and 39 °C for 1 h, the proportion of cells with a detectable signal of fluorescence was significantly higher in *hsp12.*1Δ compared to WT and *hsp12.1Δ*::*HSP12.1* strains (Fig 3B), confirming that absence of Hsp12.1 led to accumulation of intracellular ROS.

We also evaluated whether Hsp12.1 could affected the production of melanin, a well know ROS scavenger (21). However, no differences in the pigmentation of cells could be found among the evaluated strains (Fig 3C), suggesting that the defects in ROS homeostasis observed in the absence of Hsp12.1 may not involve the participation of melanin.

Lack of Hsp12.1 leads to plasma membrane sensitivity and affects *C. gattii* response to fluconazole

As Hsp12 from *S. cerevisiae* and *C. albicans* act as membrane chaperones, we evaluated whether this could be a conserved role for *C. gattii* Hsp12. Spotting plate assays employing the plasma membrane stressor SDS revealed that *hsp12.1Δ* and *hsp12.1Δ/hsp12.2Δ* double mutant strains displayed a drastic growth impairment compared to WT and reconstituted strains (Fig 4A). Considering the possible plasma membrane defect in the *hsp12.1Δ* mutant, we also evaluated its growth fitness in the presence of osmotic stressors (sorbitol and sodium chloride), as well as cell wall stressors (congo red and calcofluor white). However, no differences in the sensitivity to these stressors could be found in the mutant strain (Fig 4B). Moreover, we evaluated the sensitivity of cells lacking *HSP12.1* to antifungal agents know to target the fungal cell membrane as main target (22, 23), as amphotericin B and fluconazole. Using spotting plate assays, we found a slight decrease in the growth rate of *hsp12.1Δ* mutant only in fluconazole (Fig 4C), which was also confirmed using a fluconazole disk diffusion method where *hsp12.1*Δ mutant (37 mm) displayed larger inhibition zone than WT strain (32 mm) (data not

shown). Collectively, these results confirm that cells lacking Hsp12.1 also display defects in cell membrane.

Hsp12.1 absence alters cryptococcal interaction with phagocytes and virulence

Alveolar macrophages are the first defense line against cryptococcal infection, so the fungal loads of WT, *hsp12.1Δ*, and complemented strains were evaluated after interaction with macrophage-like cells. INF-γ and LPS-primed J774.16 macrophages displayed lower cryptococcal loads after 2h and 24h of incubation with *hsp12.1Δ* strain compared to WT and complemented strains (Fig 5A). In order to evaluate the mechanisms by which cells lacking *HSP12.1* displayed decreased association with macrophages, we evaluated important modulators of immune cells: cryptococcal capsular size and concentration of secreted polysaccharide (GXM). We could not found any significant differences in such virulence determinants when comparing *hsp12.*1Δ to WT and *hsp12.*1*Δ*::*HSP12.1* strains (Fig 5B).

As Hsp12.1 alters the outcome from the interaction between *C. gattii* and macrophages, we sought to determine if Hsp12.1 influences the cryptococcal virulence. Using a non-mammalian host (*Tenebrio molitor* larvae), we found that the *hsp12.1Δ* has a hypovirulent phonotype, which returned to the WT levels by the reintroduction of the *HSP12.1* gene. However, the virulence of the *hsp12.1Δ/hsp12.2Δ* double mutant strain did not differ from the WT (Fig 5C).

DISCUSSION

During their distinct lifestyles, cryptococcal cells may found distinct types of stresses, being the infection of mammalian host possibly the major harsh condition to the yeast (24). To cope with these situations, cryptococcal cells have to develop a response to adapt their metabolism (25), and Hsps could play a central role in this process. These proteins belongs to a remarkable family conserved from yeasts to humans with very diversified functions, including the role in response to several stresses (8). Despite the well characterized mechanisms associated with the distinct classes of Hsps, little is known about Hsp12 and its function in pathogenic fungi. Three lines of evidence allow us to conclude that Hsp12.1 is a component of the stress response in *C. gattii*: (i) *HSP12.1* displayed higher expression after a heat shock condition; (ii) null mutants displayed hypersensitivity to membrane stressors as SDS and fluconazole; and (iii) cells lacking *HSP12.1* are hypovirulent in a non-mammalian model of cryptococcosis.

The higher expression of cryptococcal *HSP12.1* under heat shock conditions follows the pattern observed in its orthologs from *S. cerevisiae*, *Schizosaccharomyces pombe*, and *C. albicans* (17, 20, 26). The Hsp12 from *C. albicans* was already characterized as a component of the High Osmolarity Glycerol (HOG) – MAPK pathway (17), a signaling cascade known to regulate the expression of genes driven by multiple environmental stress conditions (27–29). Calcineurin pathway is highly conserved among eukaryotic cells (30). It is a central hub to govern Ca^{2+} signaling cascades and it is also linked to stress response, elevated temperature growth and virulence in *Cryptococcus* (31). The calcineurin-Crz1 stress

response transcriptional network was determined in *C. neoformans*. After thermal stress, both *HSP12* genes from *C. neoformans* were upregulated in a Crz1 independent way by calcineurin pathway (32). We found that *C. gattii* cells lacking *HSP12.1* displayed hypersensitivity to FK506, a calcineurin inhibitor. This relationship reinforces the Hsp12.1 association to stress responses, as calcineurin inhibition leads to an impaired stress response (31).

Despite this increased sensitivity of cells lacking *HSP12.1* to pharmacological inhibition of calcineurin, the susceptibility to growth at 37 °C or to heat shock stress was not altered. This contrasts with the phenotype observed for Hsp12.1 ortholog in *S. cerevisiae*, whose absence led to a drastic reduction in the survival at such temperature and after heat shock (33). This suggest that cryptococcal cells rely on a much complex mechanism to cope with thermal stress.

A striking feature of cryptococcal cells lacking *HSP12.1* is the higher intracellular levels of ROS and the higher sensitivity to hydrogen peroxide. These phenotypes suggest a redox balance impairment in the *hsp12.1Δ* strain. In addition, inactivation of *HSP12.2* does not lead to oxidative stress imbalance, and the double mutant strain displayed similar phenotypes to the *hsp12.1* null mutant strain in H_2O_2 presence. This suggest that the HSP12.2, regardless of being a *HSP12.1* paralogue, did not display a redundant function in *C. gattii* cells.

C. gattii HSP12.1 inactivation also leaded to cell membrane stressor hypersensitivity. The Hsp12 orthologs from *S. cerevisiae* and *C. albicans* have their function linked to plasma membrane stabilization (16, 17, 33, 34). Also, *S. cerevisiae* Hsp12 is only active when associated to the plasma membrane, where it folds into

an alpha helix structure due to its association with long chain phospholipids (16). Absence of Hsp12 orthologs in *S. cerevisiae* and *C. albicans* also led to SDS hypersensitivity, suggesting that Hsp12 activity is conserved in *C. gattii* (17, 35, 36). However, a drastic reduction of growth could be only observed when the two paralogs were inactivated in *C. albicans*, unlike that *HSP12.1* disruption alone in *C. gattii* was enough to induce these changes in SDS and H_2O_2, and the double mutant had the same phenotype as *hsp12.1*Δ null strain. Therefore, we hypothesize that Hsp12.1 keep its function as a plasma membrane stabilizer while Hsp12.2 lost it.

HSP12.1 disrupted cells displayed lower phagocytosis index compared to WT and reconstituted cells. In consideration of the importance of capsule and capsule polysaccharide to virulence and *Cryptococcus* uptake by macrophages, we also determine capsule size and secreted GXM (37, 38). Alterations in capsule size and secreted GXM were not found between the mutant strain and WT. Pathogen-Associated Molecular Patterns (PAMPs) are recognized by Pattern Recognition Receptors (PRRs) in macrophages. Structural alteration in cell surface can promote PAMPs “camouflage”(39, 40). As the *S. cerevisiae HSP12* null strain have altered morphology after stress conditions, our first hypothesis is that alterations in cell surface due to *HSP12.1* disruption would alter the presence of PAMPs(11, 33).

In this study, Hsp12.1 was shown to be required for full cryptococcal virulence. Plasma membrane stressors hypersensitivity and an impaired redox balance are remarkable features of the *HSP12.1* disrupted strain. In addition, the expression of *HSP12* genes was extremely high during lung infection (41), a niche containing a specialized response/protection against particles and pathogens. In this way, it is

feasible to assume that cryptococcal Hsp12 may play a role also in virulence. This attenuated virulence in the mutant strain was certainly associated with its abnormal phenotypes. *T. molitor* immune response, as well as those of more complex organisms, is dependent of the PAMPs recognition. Release of cytotoxic reactive oxygen and nitrogen species are also a *T. molitor* approach during infection (42). Unexpectedly, the double mutant displayed virulence levels compared to those observed in WT cells, suggesting that cells evolve compensatory mechanisms to cope with the high stress found during the infection in the absence of Hsp12 proteins.

Here we characterize both *HSP12* genes of *C. gattii* showing that *HSP12.1* is linked to plasma membrane stabilization and its absence lead to redox status impairment. *HSP12.2* appears to lose its function as its disruption does not led to phenotype alterations. Also, we demonstrated that *HSP12.1* disruption influences *C. gattii* virulence.

MATERIALS AND METHODS

Fungal strains, plasmids and media

The *Cryptococcus gattii* R265 strain was routinely grown in YPD media (1% yeast extract, 2% peptone, and 2% dextrose) at 30°C and 200 rpm in rotation platform, added of 1,5% of agar when solid media was used. Plasmid pDNORNAT containing the nourseothricin marker cassette, was previously constructed by our group (43). Plasmid pJAF15 containing the hygromycin resistance marker cassette kindly given by Joseph Heitman (Duke University, Durham, NC, USA). YPD plates containing nourseothricin (100 µg/ml) were used to select *hsp12.1Δ* strain, and hygromycin (200 µg/ml) were used to select *hsp12.1::HSP12.1, HSP12.2Δ and HSP12.1Δ/HSP12.2Δ* strains.

Disrupted and complemented HSP12 strains construction

To construct the *HSP12.1* null strain, the vector pDONR-NAT-HSP12.1 was employed using DelsGate method (44). *HSP12.1* flanking sequences (1 kb each of one) were PCR-amplified and gel purified using PureLink™ Quick Gel Extraction and PCR Purification Combo Kit (Invitrogen). The plasmid pDONR-NAT and purified PCR products were mixed at equimolar ratios in a Gateway BP clonase™ II reaction (Invitrogen). The pDONR-NAT-HSP12.1 construct was linearized by I-SceI digestion prior to *C. gattii* R265 biolistic (45). For gene reconstitution of *hsp12.1Δ*, the coding region along with same 1 kb flanking regions of the deletion construct were amplified from *C. gattii* R265 DNA. The amplified fragment was purified in the same way as described above and subcloned into the EcoRV site of pJAF15. The plasmid was linearized by NotI digestion and transformed into the *hsp12.1Δ* strain as described

above. To construct *hsp12.2Δ* and *hsp12.1Δ/hsp12.2Δ* strains, the split marker method was employed (46). Flanking regions (5' and 3') of *HSP12.2* coding sequence were amplified (961 pb and 957 pb respectively), and double-joint PCR was used to fuse such fragments to the hemi-selection marker. Equimolar quantities of purified PCR products were used to transform *C. gattii* R265 and *hsp12.1*Δ strains by biolistic to generate *hsp12.2*Δ and *hsp12.1*Δ *hsp12.2*Δ, respectively.

Quantitative RT-PCR analysis

For the heat shock expression assay, WT strain was cultured overnight at standard culture condition, the cells were washed with PBS, and $1X10^7$ cells/mL were inoculated at high glucose Dulbecco's Modified Eagle Medium (DMEM). Cells were maintained for 2 h at 30 °C at 200 rpm, then the temperature was raised to 39 °C and cells were further incubated. The control group were maintained at 30 °C. After 10, 20, and 40 mins of incubation at 30 and 39 °C, the cells from each group were collected, PBS-washed and frozen in liquid nitrogen and kept at -80 °C until RNA extraction. RNA extraction with TRIzol™ reagent following the standard protocol. Total RNA samples were treated with Promega RQ1 RNase-Free DNase™ and reverse transcriptase was performed with ImProm-II Reverse transcriptase (Promega) using oligo-dT primer. qPCR analyzes were performed at Fast 7500 real-time PCR system Applied Biosystem™ platform with the following thermal cycling conditions: initial step of 95°C for 10 min followed by 50 cycles of 95°C for 15 s, 55°C for 15 s and 60°C for 60 s. Platinum SYBR green qPCR Supermix (Invitrogen) was used as a reaction mixture supplemented with 5 ρmol of each primer, and 2 µl of cDNA (4 ng) was added as template to a final volume of 20 µl. Each cDNA sample was analyzed in triplicate with each primer pair. Melting curve analysis was

performed at the end of the reaction to confirm a single PCR product. Actin was used as normalizer gene. Relative expression was determined by the $2^{-\Delta CT}$ method (47).

Spotting plate assay

The strains were grown overnight in YPD at 30°C and 200 rpm in a rotation platform. After washing with PBS, cells suspensions with densities ranging from 10^3 to 10^7 cells/mL were prepared and 3 µL of each suspension were plated in Yeast Nitrogen Base (YNB) containing 1% agar solid plates supplemented with the tested stressor at described concentration.

MIC assay

Cells were grown overnight as described, PBS washed, and 10^4 cells were inoculated in YNB media at the final volume of 200 µL per well containing the FK506 at distinct concentrations. Cells were incubated at 30 °C for 48 h and cell growth were determined using OD_{600} measurements.

Heat shock viability assay

The strains were grown and submitted at the same conditions described at the qRT-PCR analysis. Cells were diluted and then plated on YPD plates. After 48 h incubation at 30 °C, CFU were determined.

Intracellular ROS determination

Cells were grown overnight in YPD at 30 °C and 200 rpm in a rotation platform. After washing with PBS, a total of 10^7 cells were kept under agitation at 37 or 39 °C for 1 h in PBS. The cells were then pelleted and resuspended in PBS containing 10 µM DFCH-DA (Sigma™) and incubated at 37 °C with agitation for 1h. Cells were washed again with PBS and analyzed at Guava easyCyte Flow Cytometer (Merck Millipore) by measuring the green fluorescence of 5000 events.

Disk diffusion assay

YPD plates surface were inoculated with a swab dipped in a 10^7 cell suspension of the tested strain. A 25 µg-fluconazole disk (BD Biosciences) was placed into the center of the plate. The YPD plates were incubated at 30 °C for 48 h, then inhibition zone was measured.

Capsule size measurement, secreted GXM quantification and supernatant melanin determination

Capsule size was determined in capsule induced cells and final measurements were presented and analyzed as capsule size/cell diameter ratios, as previously published elsewhere (48). Secreted GXM was quantified by ELISA (49). To induce melanin production, 4 x 10^6 cells/mL were incubated at 30 °C and 200 rpm agitation in minimum media supplemented with 1 mM of L-DOPA for 72 h, protected from light. The media was centrifuged to remove cells and the supernatant absorbance at 400 nm were determined.

Phagocytosis index assay and *Tenebrio molitor* virulence assay

In 96-well culture plates, 10^5 J774.16 cells were seeded and activated overnight with addition of Sigma™ recombinant murine IFN-γ (100 U/ml) and Sigma™ *Escherichia coli* lipopolysaccharides (500 ng/ml). *C. gattii* strains were grown in YPD at 30°C and 200 rpm in a rotation platform. After washing with PBS, a total of 1X10^6 cells were opsonized with anti-GXM 18B7 antibody (1 µg/mL 37 °C for 1 h) in PBS. These cells were added to macrophage containing plates at a MOI of 1:10. Macrophage-*Cryptococcus* containing plates were incubated at 37 °C, 5% CO_2. After 2 or 24 h, macrophage cells were PBS washed 3 times to remove non-internalized *Cryptococcus* cells. Macrophages were lysed with addition of cold 0.1% Triton X-

100 in water and plated on YPD media. YPD plates were incubated at 30 °C for 48 h and CFU was determined. Virulence in *T. molitor* was performed as described by de Souza and collaborators, using an inoculum of 10^6 cells of each strain per larva and incubated at 37 °C for up to 10 days (50). Mortality was scored each day and survival curves were analyzed using Kaplan–Meier in GraphPad Prism software (version 8.0.2).

REFERENCES

1. Rajasingham R, Smith RM, Park BJ, Jarvis JN, Govender NP, Chiller TM, Denning DW, Loyse A, Boulware DR. 2017. Global burden of disease of HIV-associated cryptococcal meningitis: an updated analysis. Lancet Infect Dis 17:873–881.

2. Kwon-chung KJ, Fraser JA, Doering TL, Wang ZA, Janbon G, Idnurm A, Bahn Y. 2016. *Cryptococcus neoformans* and *Cryptococcus gattii*, the Etiologic Agents of Cryptococcosis. Coid Spring Harb Perspect 1–28.

3. Lin X, Heitman J. 2006. The Biology of the *Cryptococcus neoformans* Species Complex . Annu Rev Microbiol 60:69–105.

4. Perfect JR. 2005. *Cryptococcus neoformans*: A sugar-coated killer with designer genes. FEMS Immunol Med Microbiol 45:395–404.

5. May RC, Stone NRH, Wiesner DL, Bicanic T, Nielsen K. 2016. *Cryptococcus*: From environmental saprophyte to global pathogen. Nat Rev Microbiol. Nature Publishing Group.

6. Kwon-Chung KJ, Sorrell TC, Dromer F, Fung E, Levitz SM. 2000. Cryptococcosis: Clinical and biological aspects. Med Mycol 38:205–213.

7. Brown SM, Campbell LT, Lodge JK. 2007. *Cryptococcus neoformans*, a fungus under stress. Curr Opin Microbiol 10:320–325.

8. Tiwari S, Thakur R, Shankar J. 2015. Role of Heat-Shock Proteins in Cellular Function and in the Biology of Fungi. Biotechnol Res Int 2015:1–11.

9. Hartl FU, Bracher A, Hayer-Hartl M. 2011. Molecular chaperones in protein folding and proteostasis. Nature 475:324–332.

10. Jee H. 2016. Size dependent classification of heat shock proteins: a mini-review. J Exerc Rehabil 12:255–259.

11. Silveira CP, Piffer AC, Kmetzsch L, Fonseca FL, Soares DA, Staats CC, Rodrigues ML, Schrank A, Vainstein MH. 2013. The heat shock protein (Hsp) 70 of *Cryptococcus neoformans* is associated with the fungal cell surface and influences the interaction between yeast and host cells. Fungal Genet Biol 60:53–63.

12. Eastman AJ, He X, Qiu Y, Davis MJ, Vedula P, Lyons DM, Park Y-D, Hardison SE, Malachowski AN, Osterholzer JJ, Wormley FL, Williamson PR, Olszewski MA. 2015. Cryptococcal Heat Shock Protein 70 Homolog Ssa1 Contributes to Pulmonary Expansion of *Cryptococcus neoformans* during the Afferent Phase of the Immune Response by Promoting Macrophage M2 Polarization . J Immunol 194:5999–6010.

13. Kakeya H, Udono H, Ikuno N, Yamamoto Y, Mitsutake K, Miyazaki T, Tomono K, Koga H, Tashiro T, Nakayama E, Kohno S. 1997. A 77-kilodalton protein of *Cryptococcus neoformans*, a member of the heat shock protein 70 family, is a major antigen detected in the sera of mice with pulmonary cryptococcosis. Infect Immun 65:1653–1658.

14. Chatterjee S, Tatu U. 2017. Heat shock protein 90 localizes to the surface and augments virulence factors of *Cryptococcus neoformans*. PLoS Negl Trop Dis

11:1–20.

15. Cordeiro R de A, Evangelista AJ de J, Serpa R, de Farias Marques FJ, de Melo CVS, de Oliveira JS, da Silva Franco J, de Alencar LP, de Jesus Pinheiro Gomes Bandeira T, Brilhante RSN, Sidrim JJC, Rocha MFG. 2016. Inhibition of heat-shock protein 90 enhances the susceptibility to antifungals and reduces the virulence of *Cryptococcus neoformans/ Cryptococcus gattii* species complex. Microbiol (United Kingdom) 162:309–317.

16. Herbert AP, Riesen M, Bloxam L, Kosmidou E, Wareing BM, Johnson JR, Phelan MM, Pennington SR, Lian LY, Morgan A. 2012. NMR structure of Hsp12, a protein induced by and required for dietary restriction-induced lifespan extension in yeast. PLoS One 7:1–12.

17. Fu MS, de Sordi L, Mühlschlegel FA. 2012. Functional characterization of the small heat shock protein Hsp12p from *Candida albicans*. PLoS One 7.

18. Sheth CC, Mogensen EG, Fu MS, Blomfield IC, Mühlschlegel FA. 2008. *Candida albicans* HSP12 is co-regulated by physiological CO2 and pH. Fungal Genet Biol 45:1075–1080.

19. Basenko EY, Pulman JA, Shanmugasundram A, Harb OS, Crouch K, Starns D, Warrenfeltz S, Aurrecoechea C, Stoeckert CJ, Kissinger JC, Roos DS, Hertz-Fowler C. 2018. FungiDB: An integrated bioinformatic resource for fungi and oomycetes. J Fungi 4:1–28.

20. Praekelt UM, Meacock PA. 1990. HSP12, a new small heat shock gene of *Saccharomyces cerevisiae*: Analysis of structure, regulation and function.

MGG Mol Gen Genet 223:97–106.

21. Casadevall A, Rosas AL, Nosanchuk JD. 2000. Melanin and virulence in *Cryptococcus neoformans*. Curr Opin Microbiol 3:354–358.

22. Perfect JR, Bicanic T. 2015. Cryptococcosis Diagnosis and Treatment: What Do We Know Now. Fungal Genet Biol 78:49–54.

23. Ghannoum MA, Rice LB. 1999. Antifungal agents: Mode of action, mechanisms of resistance, and correlation of these mechanisms with bacterial resistance. Clin Microbiol Rev 12:501–517.

24. Esher SK, Zaragoza O, Alspaugh JA. 2018. Cryptococcal pathogenic mechanisms: A dangerous trip from the environment to the brain. Mem Inst Oswaldo Cruz 113:1–15.

25. Hu G, Cheng PY, Sham A, Perfect JR, Kronstad JW. 2008. Metabolic adaptation in *Cryptococcus neoformans* during early murine pulmonary infection. Mol Microbiol 69:1456–1475.

26. Ahn J, Won M, Choi JH, Kyun ML, Cho HS, Park HM, Kang CM, Chung KS. 2012. Small heat-shock protein Hsp9 has dual functions in stress adaptation and stress-induced G2-M checkpoint regulation via Cdc25 inactivation in *Schizosaccharomyces pombe*. Biochem Biophys Res Commun 417:613–618.

27. Rourke SMO, Herskowitz I, Shea EKO. 2002. Yeast go the whole HOG for the hyperosmotic response. Trends Genet 18:405–412.

28. Glycerol THEH, Pathway R. 2013. Stress Signaling Pathways for the

Pathogenicity of *Cryptococcus*. Eukaryot Cell 12:1564–1577.

29. Pentland DR, Piper-brown E, Mühlschlegel FA, Gourlay CW. 2018. Ras signalling in pathogenic yeasts. Microb Cell 5:63–74.

30. Goldman A, Roy J, Bodenmiller B, Wanka S, Landry CR, Aebersold R, Cyert MS. 2014. The calcineurin signaling network evolves via conserved kinase-phosphatase modules that transcend substrate identity. Mol Cell 55:422–435.

31. Park H, Chow EWL, Fu C, Soderblom EJ, Moseley MA, Heitman J, Cardenas ME. 2016. Calcineurin Targets Involved in Stress Survival and Fungal Virulence. PLoS Pathog 12:1–28.

32. Chow EWL, Clancey SA, Billmyre RB, Averette AF, Granek A, Mieczkowski P, Cardenas ME, Heitman J. 2017. Elucidation of the calcineurin-Crz1 stress response transcriptional network in the human fungal pathogen *Cryptococcus neoformans*. PLoS Genet 13:1–29.

33. Welker S, Rudolph B, Frenzel E, Hagn F, Liebisch G, Schmitz G, Scheuring J, Kerth A, Blume A, Weinkauf S, Haslbeck M, Kessler H, Buchner J. 2010. Hsp12 Is an Intrinsically Unstructured Stress Protein that Folds upon Membrane Association and Modulates Membrane Function. Mol Cell 39:507–520.

34. Sales K, Brandt W, Rumbak E, Lindsey G. 2000. The LEA-like protein HSP 12 in *Saccharomyces cerevisiae* has a plasma membrane location and protects membranes against desiccation and ethanol-induced stress. Biochim Biophys Acta - Biomembr 1463:267–278.

463 35. Maeng S, Ko YJ, Kim GB, Jung KW, Floyd A, Heitman J, Bahn YS. 2010.
464 Comparative transcriptome analysis reveals novel roles of the ras and cyclic
465 AMP signaling pathways in environmental stress response and antifungal drug
466 sensitivity in *Cryptococcus neoformans*. Eukaryot Cell 9:360–378.

467 36. Singarapu KK, Tonelli M, Chow DC, Frederick RO, Westler WM, Markley JL.
468 2011. Structural characterization of Hsp12, the heat shock protein from
469 *Saccharomyces cerevisiae*, in aqueous solution where it is intrinsically
470 disordered and in detergent micelles where it is locally α-helical. J Biol Chem
471 286:43447–43453.

472 37. Vecchiarelli A, Monari C. 2012. Capsular Material of *Cryptococcus*
473 *neoformans*: Virulence and Much More. Mycopathologia 173:375–386.

474 38. Vecchiarelli A. 2000. Immunoregulation by capsular components of
475 *Cryptococcus neoformans*. Med Mycol 38:407–417.

476 39. Erwig LP, Gow NAR. 2016. Interactions of fungal pathogens with phagocytes.
477 Nat Rev Microbiol 14:163–176.

478 40. Campuzano A, Wormley FL. 2018. Innate immunity against *Cryptococcus*,
479 from recognition to elimination. J Fungi 4.

480 41. Ferrareze PAG, Streit RSA, Santos PR dos, Santos FM dos, Almeida RMC
481 de, Schrank A, Kmetzsch L, Vainstein MH, Staats CC. 2017. Transcriptional
482 Analysis Allows Genome Reannotation and Reveals that *Cryptococcus gattii*
483 VGII Undergoes Nutrient Restriction during Infection. Microorganisms 5:49.

484 42. Vigneron A, Jehan C, Rigaud T, Moret Y. 2019. Immune Defenses of a

485 Beneficial Pest : The Mealworm Beetle , Tenebrio molitor. Front Physiol 10.

486 43. Kmetzsch L, Staats CC, Simon E, Fonseca FL, de Oliveira DL, Sobrino L,
487 Rodrigues J, Leal AL, Nimrichter L, Rodrigues ML, Schrank A, Vainstein MH.
488 2010. The vacuolar Ca2+ exchanger vcx1 is involved in calcineurin-dependent
489 Ca2+ tolerance and virulence in *Cryptococcus neoformans*. Eukaryot Cell
490 9:1798–1805.

491 44. García-Pedrajas MD, Nadal M, Kapa LB, Perlin MH, Andrews DL, Gold SE.
492 2008. DelsGate , a robust and rapid gene deletion construction method.
493 Fungal Genet Biol 45:379–388.

494 45. Toffaletti DL, Rude TH, Johnston SA, Durack DT, Perfecrl JR. 1993. Gene
495 Transfer in *Cryptococcus neoformans* by Use of Biolistic Delivery of DNA. J
496 Bacteriol 175:1405–1411.

497 46. Jung KW, Lee KT, So YS, Bahn YS. 2018. Genetic Manipulation of
498 *Cryptococcus neoformans*. Curr Protoc Microbiol 50:1–32.

499 47. Livak KJ, Schmittgen TD. 2001. Analysis of Relative Gene Expression Data
500 Using Real-Time Quantitative PCR and the 2−ΔΔCT Method. Methods
501 25:402–408.

502 48. Reuwsaat JCV, Motta H, Garcia AWA, Vasconcelos CB, Marques BM, Oliveira
503 NK, Rodrigues J, Ferrareze PAG, Frases S, Lopes W, Barcellos VA, Squizani
504 ED, Horta JA, Schrank A, Rodrigues ML, Staats CC, Vainstein MH, Kmetzsch
505 L. 2018. A Predicted Mannoprotein Participates in *Cryptococcus gattii*
506 Capsular Structure . mSphere 3.

507 49. Casadevall A, Mukherjee J, Scharff MD. 1992. Monoclonal antibody based
508 ELISAs for cryptococcal polysaccharide. J Immunol Methods 154:27–35.

509 50. de Souza PC, Morey AT, Castanheira GM, Bocate KP, Panagio LA, Ito FA,
510 Furlaneto MC, Yamada-Ogatta SF, Costa IN, Mora-Montes HM, Almeida RS.
511 2015. *Tenebrio molitor* (Coleoptera: Tenebrionidae) as an alternative host to
512 study fungal infections. J Microbiol Methods 118:182–186.

513

514

FIGURES

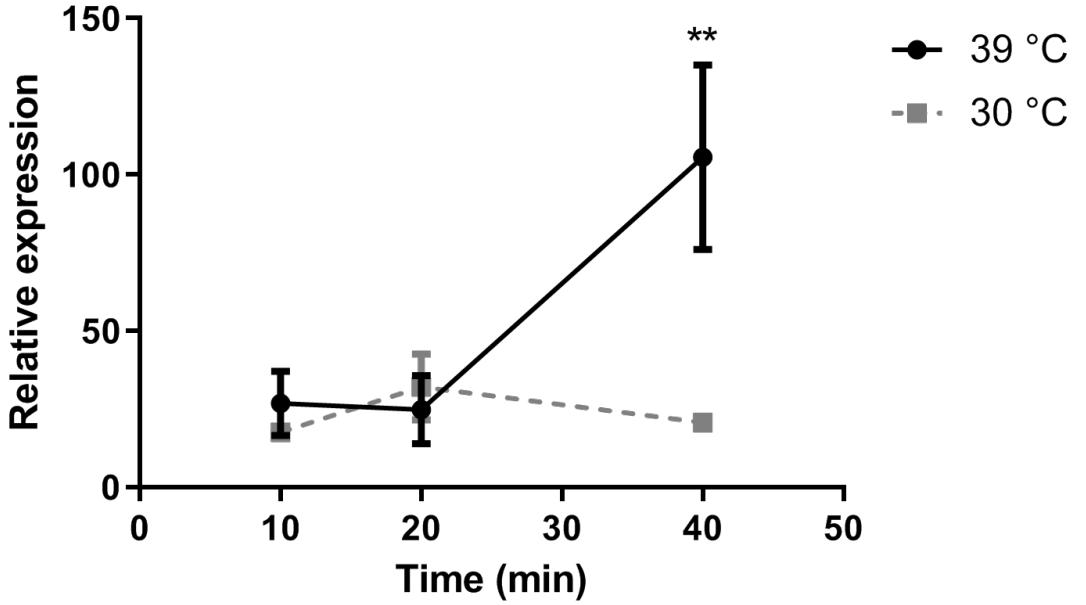
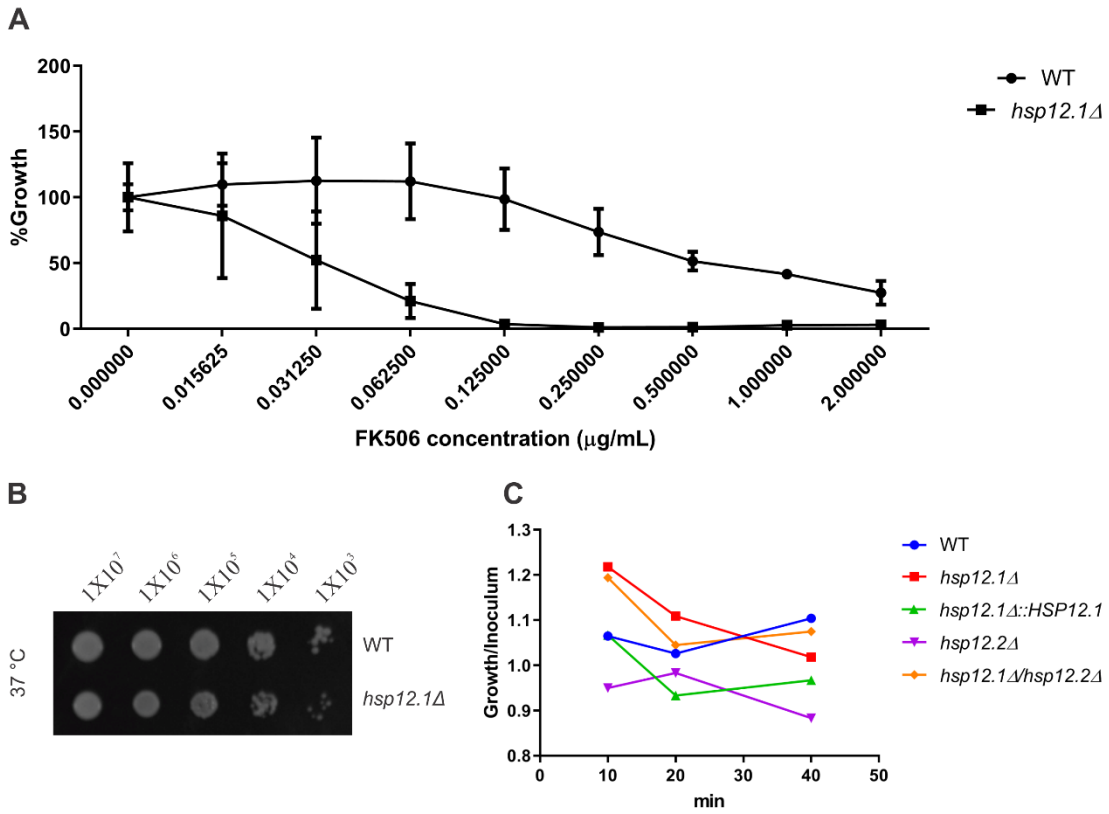


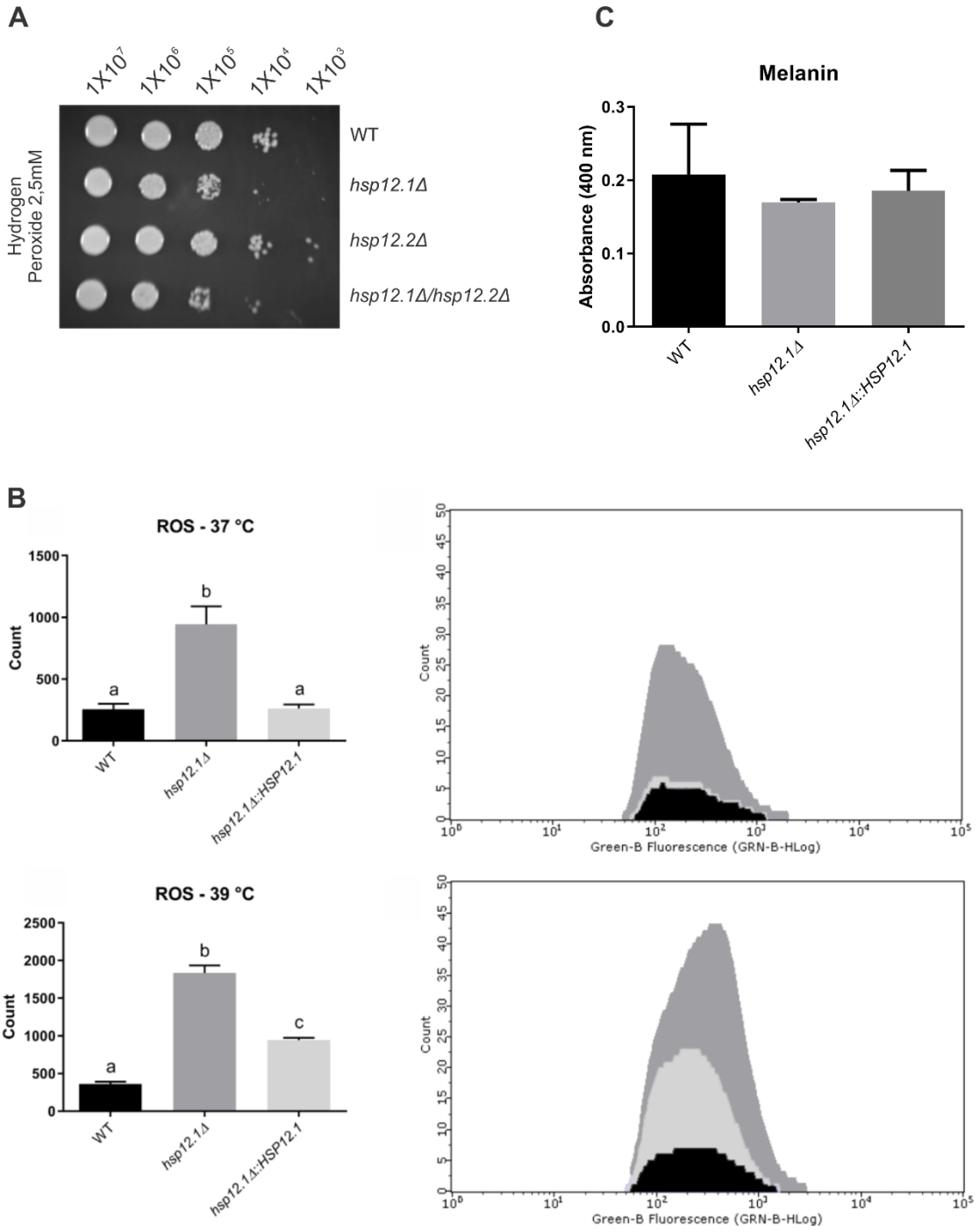
Figure 1. *HSP12* expression increases after heat shock. Time course expression analysis of *HSP12.1* after 30 °C - 39 °C heat shock condition in 10, 20 and 40 minutes. Data are shown as the mean ± standard deviation from three experimental replicates of three biological replicates. Comparisons of means were performed using unpaired t-tests and those with significant differences were marked with (**p < 0.01).



524

525 **Figure 2. *HSP12.1* disruption leads to higher sensitivity to FK506 calcineurin**
526 **inhibitor but does not affect growth at 37 °C and cell viability after heat shock.**
527 (A) Determination of FK506 minimum inhibitory concentration was performed in YNB
528 media. After 24 h of incubation, the OD_{600nm} was determined from three biological
529 replicates. Data are shown as the mean ± standard deviation (B) Spotting plate
530 assay of growth at 37 °C for 2 days. (C) Impact of heat shock upon cell viability. Cells
531 were submitted to 30 °C - 39 °C heat shock condition for 10, 20, and 40 minutes.
532 Cryptococcal cells were plated in YPD to viability analysis through colony-forming
533 unity (CFU) counts. Data are shown as the mean from three biological replicates.

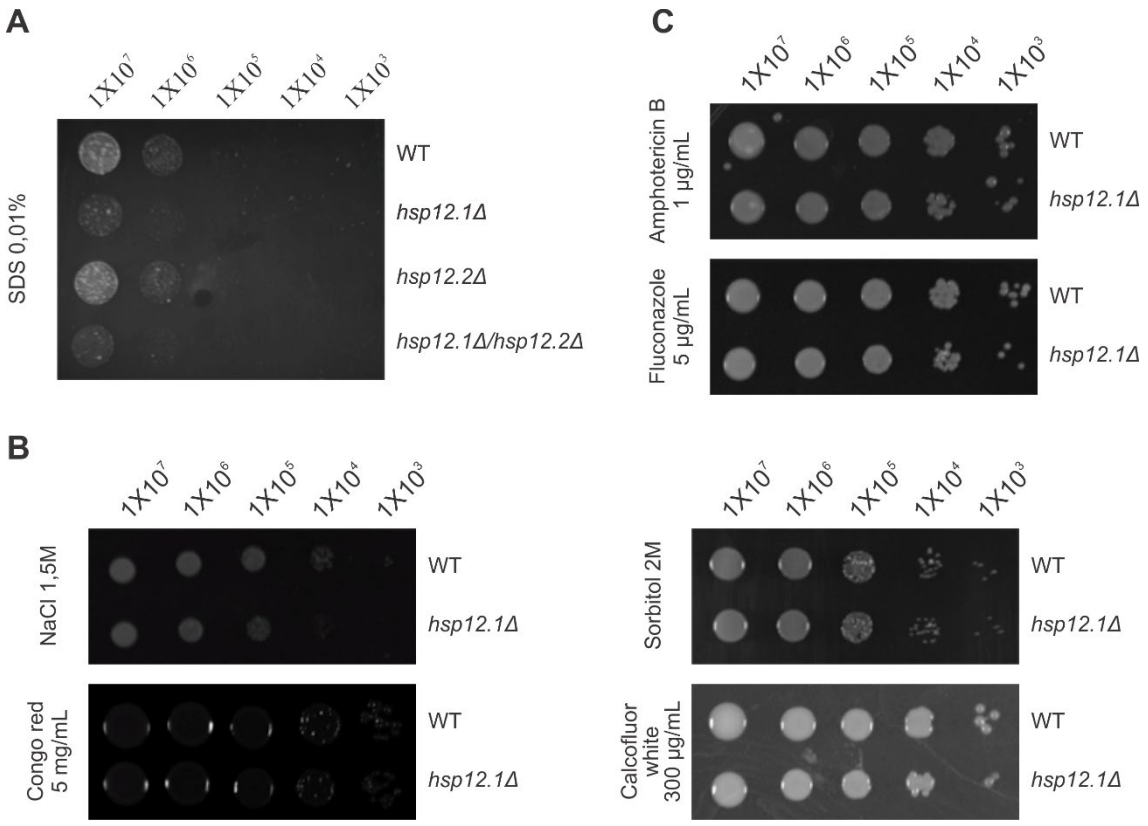
534



535

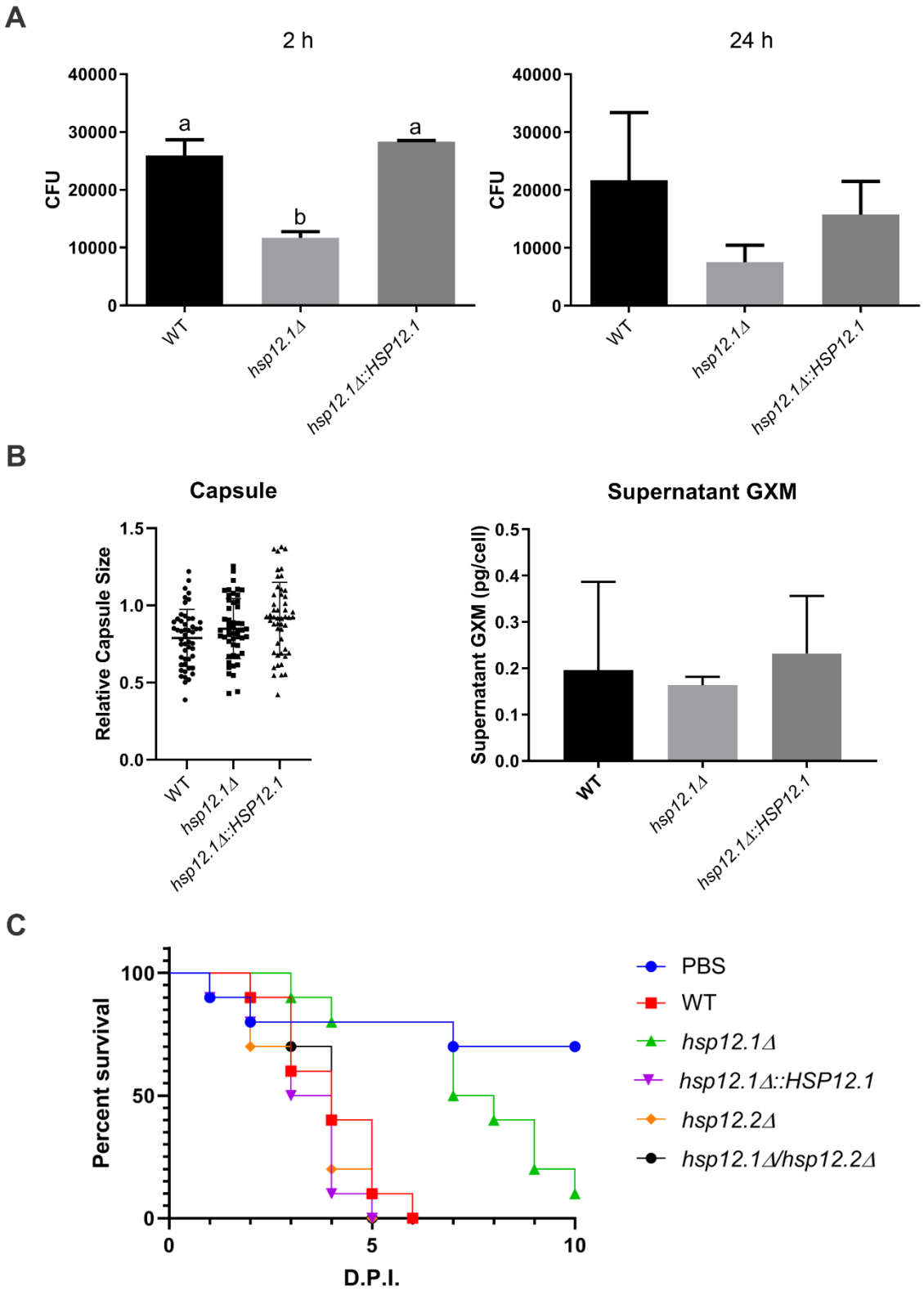
536 **Figure 3. Absence of *HSP12.1* leads to redox impairment through reactive**
537 **species accumulation despite normal melanin production.** (A) Spotting plate
538 assay in YNB added of hydrogen peroxide. (B) Intracellular ROS were evaluated by
539 flow cytometry of DFCH-DA stained cells with previous 1 h incubation at 37 °C and

39 °C. Data are shown as the mean ± standard deviation (left panels) of geometric mean fluorescence intensity (right panels) of WT, *hsp12.1Δ,* and *hsp12.1Δ::HSP12.1* strains. Comparisons of means were performed using ANOVA tests and those with significant differences were marked with distinct letters. (C) Released melanin in media was quantified using spectrophotometry (OD_{400nm}) from cultures of WT, *hsp12.1Δ,* and *hsp12.1Δ::HSP12.1* strains in conditioned media.



547

548 **Figure 4. *HSP12.1* disruption, but not *HSP12.2*, caused defects in plasma**
549 **membrane and higher susceptibility to hydrogen peroxide.** Spotting plate assay
550 was performed by plating 3 µL 10-fold serially diluted suspension of WT, *hsp12.1Δ,*
551 and *hsp12.1Δ::HSP12.1* strains in YNB agar supplemented with (A) plasma
552 membrane stressor (SDS), (B) osmotic stressors (NaCl and Sorbitol), cell wall
553 stressors (Congo red, and Calcofluor white), and (C) Antifungal drugs (Amphotericin
554 B, and Fluconazole) at the indicated concentrations.


A
2 h
24 h
CFU
40000
30000
20000
10000
0
a
b
a
WT
hsp12.1Δ
hsp12.1Δ::HSP12.1
B
Capsule
Relative Capsule Size
1.5
1.0
0.5
0.0
Supernatant GXM
Supernatant GXM (pg/cell)
0.5
0.4
0.3
0.2
0.1
0.0
C
Percent survival
100
50
0
0
5
10
D.P.I.
PBS
WT
hsp12.1Δ
hsp12.1Δ::HSP12.1
hsp12.2Δ
hsp12.1Δ/hsp12.2Δ

555

Figure 5. Hsp12.1 inactivation affects cryptococcal interaction with phagocytes and virulence. (A) J774.16 macrophage-like activated cells (IFN-γ and LPS) were incubated with opsonized WT, *hsp12.1Δ, hsp12.1Δ::HSP12.1* cells for 2 h and 24 h. Macrophages were washed to remove non-internalized cells, lysed and plated in YPD agar to CFU analysis. Data are shown as the mean ± SD. The letters a, b and c denote significant difference between means ($p<0.001$) in comparison to the other conditions. Comparisons were analyzed by one-way analysis of variance followed by Tukey's multicomparison test. (B) Relative capsule size was determined by India ink microscopy from cultures in capsule inducing medium. Supernatant GXM determination by ELISA. (C) *T. molitor* mortality curves. Larvae deaths were registered daily and the median survival days were 4 (WT), 7.5 (*hsp12.1Δ*), 3.5 (*hsp12.1Δ::HSP12.1*), 4 (*hsp12.2Δ*), and 4 (*hsp12.1Δ/hsp12.2Δ*), as evaluated by Kaplan Meier analysis.

SUPPLEMENTAL MATERIAL

Table S1. Primers used in the present work.

Primer	Sequence (5’ – 3’)	Use
ACT1 qRT-PCR F	CGGTATCGTCACAAACTGG	qRT-PCR Actin
ACT1 qRT-PCR R	GGAGCCTCGGTAAGAAGAAC	qRT-PCR Actin
CNBG_2441 qRT-PCR F	GACTGACTCTGCTGCTTCTAC	qRT-PCR *HSP12.1*
CNBG_2441 qRT-PCR R	GACTCCTGGTTGTCGTTCTT	qRT-PCR *HSP12.1*
CNBG_5853 qRT-PCR F	TTACCGACAAGGCTTCTTC	qRT-PCR *HSP12.2*
CNBG_5853 qRT-PCR R	GCATCTCCAATCTTCTGAG	qRT-PCR *HSP12.2*
CNBG_2441_5'F	AAAATAGGGATAACAGGGTAATGACCAAGGTTGGGATGCTTA	*HSP12.1* disruption
CNBG_2441_5'R	GGGGACAAGTTTGTACAAAAAAGCAGGCTATGTGTTTGTGCTGTGGTTTGG	*HSP12.1* disruption
CNBG_2441_3'F	GGGGACCACTTTGTACAAGAAAGCTGGGTAGCAGTTATCGCAACCATTGA	*HSP12.1* disruption
CNBG_2441_3'R	AAAAATTACCCTGTTATCCCTAAAAGCGGATCTGTCGCCTAT	*HSP12.1* disruption
CNBG_5853_5'F	CAGTTGTAGTTATTTCAGTCAATGC	*HSP12.2* disruption
CNBG_5853_5'R	GCTCACTGGCCGTCGTTTTACGCTTGTGTTTGAGTTTGTTGTG	*HSP12.2* disruption
CNBG_5853_3'F	CATGGTCATAGCTGTTTCCTGAAGAAGGAAATAGTCGCGTGAAG	*HSP12.2* disruption
CNBG_5853_3'R	TTCAAGAGGTTGAGAGGATTGATAC	*HSP12.2* disruption

4. Conclusões

I. A expressão de *HSP12.1* é responsiva ao choque térmico;

II. A inativação de *HSP12.1* acarreta em (a) aumento na sensibilidade à inibição da via da calcineurina por FK506; (b) aumento na sensibilidade ao estresse oxidativo; (c) acúmulo intracelular de espécies reativas de oxigênio; (d) aumento na sensibilidade ao estresse de membrana plasmática; (e) redução da taxa de fagocitose em macrófagos da linhagem J774.16;

III. A linhagem nula para *HSP12.1* apresenta fenótipo de hipovirulência em modelo invertebrado;

IV. A função de Hsp12 em *C. gattii* é de potencial chaperona da membrana plasmática, assim como em *S. cerevisiae* e *C. albicans*;

V. A inativação de *HSP12.2* não apresentou diferenças quando comparado com a linhagem selvagem nos fenótipos testados, sugerindo perda de função ou efeito compensatório por *HSP12.1*.

5. Perspectivas

I. Reproduzir os ensaios de quantificação de ROS, MIC de FK506 e taxa de fagocitose para a linhagem *hsp12.2*Δ e *hsp12.1*Δ*/hsp12.2*Δ;

II. Determinar a localização de Hsp12.1 e Hsp12.2 empregando a imunolocalização das proteínas marcadas com HA-flag;

III. Avaliar a permeabilidade de membrana plasmática nas linhagens mutantes;

IV. Avaliar a quantidade de esteróis presente na membrana plasmática das linhagens construídas;

V. Avaliar a virulência das linhagens mutantes em camundongos da linhagem BALB/c.

6. Referências

Agustinho, D. P., Miller, L. C., Li, L. X., & Doering, T. L. (2018). Peeling the onion : the outer layers of *Cryptococcus neoformans*. *Memórias Do Instituto Oswaldo Cruz*, *113*(7), 1–8. https://doi.org/10.1590/0074-02760180040

Alves Soares, E., Lazera, M. dos S., Wanke, B., Faria Ferreira, M. de, Carvalhaes de Oliveira, R. V., Oliveira, A. G., & Coutinho, Z. F. (2019). Mortality by cryptococcosis in Brazil from 2000 to 2012: A descriptive epidemiological study. *PLOS Neglected Tropical Diseases*, *13*(7), e0007569. https://doi.org/10.1371/journal.pntd.0007569

Bukau, B., & Horwich, A. L. (1998). The Hsp70 and Hsp60 chaperone machines. *Cell*, *92*(3), 351–366. https://doi.org/10.1016/S0092-8674(00)80928-9

Burnie, J. P., Carter, T. L., Hodgetts, S. J., & Matthews, R. C. (2006). Fungal heat-shock proteins in human disease. *FEMS Microbiology Reviews*, *30*(1), 53–88. https://doi.org/10.1111/j.1574-6976.2005.00001.x

Campuzano, A., & Wormley, F. L. (2018). Innate immunity against *Cryptococcus*, from recognition to elimination. *Journal of Fungi*, *4*(1). https://doi.org/10.3390/jof4010033

Casadevall, A., Rosas, A. L., & Nosanchuk, J. D. (2000). Melanin and virulence in *Cryptococcus neoformans*. *Current Opinion in Microbiology*, *3*(4), 354–358. https://doi.org/https://doi.org/10.1016/S1369-5274(00)00103-X

Chatterjee, S., & Tatu, U. (2017). Heat shock protein 90 localizes to the surface and augments virulence factors of *Cryptococcus neoformans*. *PLoS Neglected Tropical Diseases*, *11*(8), 1–20. https://doi.org/10.1371/journal.pntd.0005836

Chen, Y., Lehman, V. N., Lewit, Y., Averette, A. F., & Heitman, J. (2013). Calcineurin Governs Thermotolerance and Virulence of *Cryptococcus gattii*. *G3 (Bethesda)*, *3*(3), 527–539. https://doi.org/10.1534/g3.112.004242

Chow, E. W. L., Clancey, S. A., Billmyre, R. B., Averette, A. F., Granek, A., Mieczkowski, P., … Heitman, J. (2017). Elucidation of the calcineurin-Crz1 stress response transcriptional network in the human fungal pathogen *Cryptococcus neoformans*. 1–29. https://doi.org/10.1371/journal.pgen.1006667

Cordeiro, R. de A., Evangelista, A. J. de J., Serpa, R., de Farias Marques, F. J., de Melo, C. V. S., de Oliveira, J. S., … Rocha, M. F. G. (2016). Inhibition of heat-shock protein 90 enhances the susceptibility to antifungals and reduces the virulence of *Cryptococcus neoformans/ Cryptococcus gattii* species complex. *Microbiology (United Kingdom)*, *162*(2), 309–317. https://doi.org/10.1099/mic.0.000222

Cyert, M. S. (2003). Calcineurin signaling in *Saccharomyces cerevisiae*: How yeast go crazy in response to stress. *Biochemical and Biophysical Research Communications*, *311*(4), 1143–1150. https://doi.org/10.1016/S0006-291X(03)01552-3

Dadachova, E., Bryan, R. A., Huang, X., Moadel, T., Schweitzer, A. D., Aisen, P., … Casadevall, A. (2007). Ionizing Radiation Changes the Electronic Properties of Melanin and Enhances the Growth of Melanized Fungi. *PLoS ONE*, *2*(5), e457. https://doi.org/10.1371/journal.pone.0000457

Dickson, R. P., Erb-Downward, J. R., Martines, F. J., & Huffnagle, G. B. (2016). The Microbiome and the Respiratory Tract. *Annual Review of Physiology*,

78, 481–504. https://doi.org/10.1146/annurev-physiol-021115-105238

Eastman, A. J., He, X., Qiu, Y., Davis, M. J., Vedula, P., Lyons, D. M., … Olszewski, M. A. (2015). Cryptococcal Heat Shock Protein 70 Homolog Ssa1 Contributes to Pulmonary Expansion of *Cryptococcus neoformans* during the Afferent Phase of the Immune Response by Promoting Macrophage M2 Polarization . *The Journal of Immunology*, *194*(12), 5999–6010. https://doi.org/10.4049/jimmunol.1402719

Ellis, D. H., & Pfeiffer, T. J. (1990). Natural habitat of *Cryptococcus neoformans var. gattii*. *Journal of Clinical Microbiology*, *28*(7), 1642–1644.

Erwig, L. P., & Gow, N. A. R. (2016). Interactions of fungal pathogens with phagocytes. *Nature Reviews Microbiology*, *14*(3), 163–176. https://doi.org/10.1038/nrmicro.2015.21

Esher, S. K., Zaragoza, O., & Alspaugh, J. A. (2018). Cryptococcal pathogenic mechanisms: A dangerous trip from the environment to the brain. *Memorias Do Instituto Oswaldo Cruz*, *113*(7), 1–15. https://doi.org/10.1590/0074-02760180057

Fan, W., Kraus, P. R., Boily, M. J., & Heitman, J. (2005). *Cryptococcus neoformans* gene expression during murine macrophage infection. *Eukaryotic Cell*, *4*(8), 1420–1433. https://doi.org/10.1128/EC.4.8.1420-1433.2005

Feldmesser, M., Kress, Y., Novikoff, P., & Casadevall, A. (2000). *Cryptococcus neoformans* Is a Facultative Intracellular Pathogen in Murine Pulmonary Infection. *Infection and Immunity*, *68*(7), 4225–4237.

Fromtling, R. A., Shadomy, H. J., & Jacobson, E. S. (1982). Decreased virulence in stable, acapsular mutants of *Cryptococcus neoformans*. *Mycopathologia*, *79*(1), 23–29. https://doi.org/10.1007/BF00636177

Fu, M. S., de Sordi, L., & Mühlschlegel, F. A. (2012). Functional characterization of the small heat shock protein Hsp12p from *Candida albicans*. *PLoS ONE*, *7*(8). https://doi.org/10.1371/journal.pone.0042894

García-Rodas, R., & Zaragoza, O. (2012). Catch me if you can: phagocytosis and killing avoidance by *Cryptococcus neoformans*. *FEMS Immunology and Medical Microbiology*, *64*, 147–161. https://doi.org/10.1111/j.1574-695X.2011.00871.x

Giles, S. S., Zaas, A. K., Reidy, M. F., Perfect, J. R., & Wright, J. R. (2007). *Cryptococcus neoformans* is resistant to Surfactant protein A mediated host defense mechanisms. *PLoS ONE*, *2*(12), 1–7. https://doi.org/10.1371/journal.pone.0001370

Glasser, J. R., & Mallampalli, R. K. (2012). Surfactant and its role in the pathobiology of pulmonary infection. *Microbes and Infection*, *14*(1), 17–25. https://doi.org/10.1016/j.micinf.2011.08.019

Hartl, F. U., Bracher, A., & Hayer-Hartl, M. (2011). Molecular chaperones in protein folding and proteostasis. *Nature*, *475*(7356), 324–332. https://doi.org/10.1038/nature10317

Haslbeck, M., & Vierling, E. (2015). A First Line of Stress Defense: Small Heat Shock Proteins and their function in protein homeostasis. *Journal of Molecular Biology*, *427*(7), 1537–1548. https://doi.org/10.1016/j.jmb.2015.02.002.A

Hu, G., Cheng, P. Y., Sham, A., Perfect, J. R., & Kronstad, J. W. (2008). Metabolic adaptation in *Cryptococcus neoformans* during early murine pulmonary infection. *Molecular Microbiology*, *69*(6), 1456–1475. https://doi.org/10.1111/j.1365-2958.2008.06374.x

Kakeya, H., Udono, H., Ikuno, N., Yamamoto, Y., Mitsutake, K., Miyazaki, T., … Kohno, S. (1997). A 77-kilodalton protein of *Cryptococcus neoformans*, a member of the heat shock protein 70 family, is a major antigen detected in the sera of mice with pulmonary cryptococcosis. *Infection and Immunity*, *65*(5), 1653–1658.

Kakeya, H., Udono, H., Maesaki, S., Sasaki, E., Kawamura, S., Hossain, M. A., … Kohno, S. (1999). Heat shock protein 70 (hsp70) as a major target of the antibody response in patients with pulmonary cryptococcosis. *Clinical and Experimental Immunology*, *115*(3), 485–490. https://doi.org/10.1046/j.1365-2249.1999.00821.x

Kidd, S. E., Hagen, F., Tscharke, R. L., Huynh, M., Bartlett, K. H., Fyfe, M., … Meyer, W. (2004). A rare genotype of *Cryptococcus gattii* caused the cryptococcosis outbreak on Vancouver Island (British Columbia, Canada). *Proceedings of the National Academy of Sciences of the United States of America*, *101*(49), 17258–17263. https://doi.org/10.1073/pnas.0402981101

Kregel, K. C. (2002). Heat shock proteins: modifying factors in physiological stress responses and acquired thermotolerance. *Journal of Applied Physiology*, *92*(5), 2177–2186. https://doi.org/10.1152/japplphysiol.01267.2001

Kronstad, J., Saikia, S., Nielson, E. D., Kretschmer, M., Jung, W., Hu, G., …

Attarian, R. (2012). Adaptation of *Cryptococcus neoformans* to mammalian hosts: Integrated regulation of metabolism and virulence. *Eukaryotic Cell*, *11*(2), 109–118. https://doi.org/10.1128/EC.05273-11

Kronstad, J. W., Attarian, R., Cadieux, B., Choi, J., D'Souza, C. A., Griffiths, E. J., … Wang, J. (2011). Expanding fungal pathogenesis: *Cryptococcus* species break out of the opportunistic box. *Nature Reviews Microbiology*, *9*(3), 193–203. https://doi.org/10.1038/nrmicro2522

Kwon-chung, K. J., & Bennett, J. E. (1984). Epidemiologic differences between the two varieties of *Cryptococcus neoformans*. *American Journal of Epidemiology*, *120*(1), 123–130. https://doi.org/10.1093/oxfordjournals.aje.a113861

Lamoth, F., Juvvadi, P. R., & Steinbach, W. J. (2016). Heat shock protein 90 (Hsp90): A novel antifungal target against *Aspergillus fumigatus*. *Critical Reviews in Microbiology*, *42*(2), 310–321. https://doi.org/10.3109/1040841X.2014.947239

Lin, X., & Heitman, J. (2006). The Biology of the *Cryptococcus neoformans* Species Complex . *Annual Review of Microbiology*, *60*(1), 69–105. https://doi.org/10.1146/annurev.micro.60.080805.142102

Liu, L., Tewari, R. P., & Williamson, P. R. (1999). Laccase protects *Cryptococcus neoformans* from antifungal activity of alveolar macrophages. *Infection and Immunity*, *67*(11), 6034–6039.

Maeng, S., Ko, Y. J., Kim, G. B., Jung, K. W., Floyd, A., Heitman, J., & Bahn, Y. S. (2010). Comparative transcriptome analysis reveals novel roles of the ras and cyclic AMP signaling pathways in environmental stress response and

antifungal drug sensitivity in *Cryptococcus neoformans*. *Eukaryotic Cell*, *9*(3), 360–378. https://doi.org/10.1128/EC.00309-09

Mansour, M. K., Reedy, J. L., Tam, J. M., & Vyas, J. M. (2014). Macrophage *Cryptococcus* interactions: an update. *Current Fungal Infection Reports*, *8*(1), 109–115. https://doi.org/10.1007/s12281-013-0165-7

Maziarz, E. K., & Perfect, J. R. (2016). Cryptococcosis. *Infectious Disease Clinics of North America*, *30*(1), 179–206. https://doi.org/10.1016/j.idc.2015.10.006.Cryptococcosis

McFadden, D. C., De Jesus, M., & Casadevall, A. (2006). The physical properties of the capsular polysaccharides from *Cryptococcus neoformans* suggest features for capsule construction. *Journal of Biological Chemistry*, *281*(4), 1868–1875. https://doi.org/10.1074/jbc.M509465200

McFadden, D. C., Fries, B. C., Wang, F., & Casadevall, A. (2007). Capsule structural heterogeneity and antigenic variation in *Cryptococcus neoformans*. *Eukaryotic Cell*, *6*(8), 1464–1473. https://doi.org/10.1128/EC.00162-07

Monari, C., Bistoni, F., Casadevall, A., Pericolini, E., Pietrella, D., Kozel, T. R., & Vecchiarelli, A. (2005). Glucuronoxylomannan, a Microbial Compound, Regulates Expression of Costimulatory Molecules and Production of Cytokines in Macrophages. *The Journal of Infectious Diseases*, *191*(1), 127–137. https://doi.org/10.1086/426511

O'Meara, T. R., & Alspaugh, J. A. (2012). The *Cryptococcus neoformans* capsule: A sword and a shield. *Clinical Microbiology Reviews*, *25*(3), 387–408. https://doi.org/10.1128/CMR.00001-12

Odom, A., Muir, S., Lim, E., Toffaletti, D. L., Perfect, J., & Heitman, J. (1997). Calcineurin is required for virulence of *Cryptococcus neoformans*. *EMBO J.*, *16*(10), 2576–2589. https://doi.org/10.1093/emboj/16.10.2576

Park, H., Chow, E. W. L., Fu, C., Soderblom, E. J., Moseley, M. A., Heitman, J., & Cardenas, M. E. (2016). Calcineurin Targets Involved in Stress Survival and Fungal Virulence. *PLoS Pathogens*, *12*(9), 1–28. https://doi.org/10.1371/journal.ppat.1005873

Praekelt, U. M., & Meacock, P. A. (1990). HSP12, a new small heat shock gene of *Saccharomyces cerevisiae*: Analysis of structure, regulation and function. *MGG Molecular & General Genetics*, *223*(1), 97–106. https://doi.org/10.1007/BF00315801

Qiu, X. B., Shao, Y. M., Miao, S., & Wang, L. (2006). The diversity of the DnaJ/Hsp40 family, the crucial partners for Hsp70 chaperones. *Cellular and Molecular Life Sciences*, *63*(22), 2560–2570. https://doi.org/10.1007/s00018-006-6192-6

Rajasingham, R., Smith, R. M., Park, B. J., Jarvis, J. N., Govender, N. P., Chiller, T. M., … Boulware, D. R. (2017). Global burden of disease of HIV-associated cryptococcal meningitis: an updated analysis. *The Lancet Infectious Diseases*, *17*(8), 873–881. https://doi.org/10.1016/S1473-3099(17)30243-8

Robbins, N., Uppuluri, P., Nett, J., Rajendran, R., Ramage, G., Lopez-Ribot, J. L., … Cowen, L. E. (2011). Hsp90 governs dispersion and drug resistance of fungal biofilms. *PLoS Pathogens*, *7*(9), e1002257. https://doi.org/10.1371/journal.ppat.1002257

Rodrigues, M. L., Nakayasu, E. S., Oliveira, D. L., Nimrichter, L., Nosanchuk, J.

D., Almeida, I. C., & Casadevall, A. (2008). Extracellular vesicles produced by *Cryptococcus neoformans* contain protein components associated with virulence. *Eukaryotic Cell*, *7*(1), 58–67. https://doi.org/10.1128/EC.00370-07

Sales, K., Brandt, W., Rumbak, E., & Lindsey, G. (2000). The LEA-like protein HSP 12 in *Saccharomyces cerevisiae* has a plasma membrane location and protects membranes against desiccation and ethanol-induced stress. *Biochimica et Biophysica Acta - Biomembranes*, *1463*(2), 267–278. https://doi.org/10.1016/S0005-2736(99)00215-1

Sheth, C. C., Mogensen, E. G., Fu, M. S., Blomfield, I. C., & Mühlschlegel, F. A. (2008). *Candida albicans* HSP12 is co-regulated by physiological CO2 and pH. *Fungal Genetics and Biology*, *45*(7), 1075–1080. https://doi.org/10.1016/j.fgb.2008.04.004

Silveira, C. P., Piffer, A. C., Kmetzsch, L., Fonseca, F. L., Soares, D. A., Staats, C. C., … Vainstein, M. H. (2013). The heat shock protein (Hsp) 70 of *Cryptococcus neoformans* is associated with the fungal cell surface and influences the interaction between yeast and host cells. *Fungal Genetics and Biology*, *60*, 53–63. https://doi.org/10.1016/j.fgb.2013.08.005

Steenbergen, J. N., Shuman, H. A., & Casadevall, A. (2001). *Cryptococcus neoformans* interactions with amoebae suggest an explanation for its virulence and intracellular pathogenic strategy in macrophages. *Proceedings of the National Academy of Sciences of the United States of America*, *98*(26), 15245–15250. https://doi.org/10.1073/pnas.261418798

Tiwari, S., Thakur, R., & Shankar, J. (2015). Role of Heat-Shock Proteins in Cellular Function and in the Biology of Fungi. *Biotechnology Research*

International, *2015*, 1–11. https://doi.org/10.1155/2015/132635

Van De Wetering, J. K., Coenjaerts, F. E. J., Vaandrager, A. B., Van Golde, L. M. G., & Batenburg, J. J. (2004). Aggregation of *Cryptococcus neoformans* by Surfactant Protein D Is Inhibited by Its Capsular Component Glucuronoxylomannan. *Infection and Immunity*, *72*(1), 145–153. https://doi.org/10.1128/IAI.72.1.145-153.2004

Vecchiarelli, A. (2000). Immunoregulation by capsular components of *Cryptococcus neoformans*. *Medical Mycology*, *38*(6), 407–417. https://doi.org/10.1080/mmy.38.6.407.417

Verghese, J., Abrams, J., Wang, Y., & Morano, K. A. (2012). Biology of the Heat Shock Response and Protein Chaperones: Budding Yeast (Saccharomyces cerevisiae) as a Model System. *Microbiology and Molecular Biology Reviews*, *76*(2), 115–158. https://doi.org/10.1128/mmbr.05018-11

Wang, Y., & Casadevall, A. (1994). Susceptibility of melanized and nonmelanized *Cryptococcus neoformans* to nitrogen- and oxygen-derived oxidants. *Infection and Immunity*, *62*(7), 3004–3007.

Wang, Yulin, Aisen, P., & Casadevall, A. (1995). *Cryptococcus neoformans* Melanin and Virulence : Mechanism of Action. *Infection and Immunity*, *63*(8), 3131–3136.

Welker, S., Rudolph, B., Frenzel, E., Hagn, F., Liebisch, G., Schmitz, G., … Buchner, J. (2010). Hsp12 Is an Intrinsically Unstructured Stress Protein that Folds upon Membrane Association and Modulates Membrane Function. *Molecular Cell*, *39*(4), 507–520. https://doi.org/10.1016/j.molcel.2010.08.001

Xie, J. L., Polvi, E. J., Shekhar-Guturja, T., & Cowen, L. E. (2014). Elucidating drug resistance in human fungal pathogens. *Future Microbiology*, *9*(4), 523–542. https://doi.org/10.2217/fmb.14.18

Yang, D. H., Jung, K. W., Bang, S., Lee, J. W., Song, M. H., Floyd-Averette, A., … Bahn, Y. S. (2017). Rewiring of signaling networks modulating thermotolerance in the human pathogen *Cryptococcus neoformans*. In *Genetics* (Vol. 205). https://doi.org/10.1534/genetics.116.190595

Zhu, X., & Williamson, P. R. (2004). Role of laccase in the biology and virulence of *Cryptococcus neoformans*. *FEMS Yeast Research*, *5*(4), 1–10. https://doi.org/10.1016/j.femsyr.2004.04.004