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IMPORTÂNCIA DO MANGANÊS PARA VIRULÊNCIA DE *Cryptococcus gattii*

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“Nothing of me is original. I am the combined effort of everyone I’ve ever known” – Chuck Palahniuk

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SUMÁRIO

LISTA DE ABREVIATURAS, SÍMBOLOS E UNIDADES

ACDP – *Ancient conserved domain proteins*

AFLP – *Amplified fragment length polymorphism*

AIDS – Síndrome da imunodeficiência adquirida, do inglês *acquired immunodefiency syndrome*

Ca^{+2} - Cálcio

CCP – Citocromo C Peroxidase

COX6B – Subunidade 6B do citocromo C oxidase

DNA – Ácido desoxirribonucleico

EVs – Vesículas extracelulares, do inglês *extracellular vesicles*

Fe^{+2} - Ferro

GalXM - Galactoxilomanana

GXM - Glicuronoxilomanana

H^{+} - Próton

HIV – Vírus da imunodeficiência humana, do inglês *human deficiency virus*

HSP70 – Proteína de choque térmico 70

IL-1β – Interleucina 1 beta

IL-10 – Interleucina dez

IL-17 – Interleucina dezessete

IL-17A – Interleucina dezessete A

Il-22 – Interleucina vinte e dois

IL-6 – Interleucina seis

M1 – Macrófago ativado classicamente

M2 – Macrófago ativado alternativamente

MC – *Mitochondrial carrier*

MFS – *Major facilitator superfamily*

MHC – *Major histocompatibility complex*

Mn^{+2} - Manganês

mRNA – RNA mensageiro

Nramp – *Natural resistance-associated macrophage protein*

NETs – *Neutrophil extracelular traps*

NO – Óxido nítrico

PAMPs – Padrões moleculares associados a patógenos, do inglês p*athogen-associated molecular patterns*

PCR – Reação em cadeia da polimerase, do inglês *polymerase chain reaction*

Plb1 – Fosfolipase B1

pH – Potencial hidrogeniônico

PLP – Piroxidal fosfato

RNA – Ácido ribonucleico

ROS – Espécies reativas de oxigênio, do inglês *reactive oxygen species*

SLC11 – *Solute carrier Family 11*

SNC – Sistema nervosa central

SOD1 – Cobre/zinco superóxido dismutase

SOD2 – Manganês superóxido dismutase

Th1 – Linfócito T auxiliar tipo 1

Th17 – Linfócito T auxiliar tipo 17

Th2 – Linfócito T auxiliar tipo 2

TNF-α – Fator de necrose tumoral alfa

ZIP – *Zrt/irt–like protein*

RESUMO

Cryptococcus gattii é um dos agentes etiológicos da criptococose, doença que inicia nos pulmões, com possibilidade de disseminação para o sistema nervoso central e outros órgãos. Os macrófagos alveolares representam a primeira linha de defesa do hospedeiro contra patógenos respiratórios, determinando o desfecho da infecção. Dentre as estratégias utilizadas pelo hospedeiro para se defender contra microrganismos invasores, destaca-se a imunidade nutricional, que consiste no controle da biodisponibilidade de micronutrientes no sítio de infecção. Manganês constitui um alvo interessante de sequestro visto que é essencial para o funcionamento do fungo e progressão da infecção. Embora sistemas que regulam a homeostase de manganês tenham sido bem descritos na levedura modelo *Saccharomyces cerevisiae*, há pouca informação a respeito de patógenos fúngicos e sua relação com a virulência. No presente trabalho foram caracterizados dois sistemas de importação de manganês na levedura patogênica *C. gattii*. O gene *ZIP3* de *C. gattii* é um ortólogo direto de *ATX2* de *S. cerevisiae*, que codifica um transportador localizado no complexo de Golgi e é responsável pela extrusão de manganês dessa organela para o citoplasma. A ausência do produto desse gene levou a um aumento da tolerância a manganês, aumento da sensibilidade a espécies reativas de oxigênio e alteração da expressão de sistemas redox na célula. Ademais, células ausentes em Zip3 apresentaram redução na virulência em modelo não mamífero de infecção, corroborando com alterações em determinantes de virulência clássicos nessa levedura, como deposição do pigmento melanina, secreção do polissacarídeo glicuronoxilomanana e perfil alterado de secreção de vesículas extracelulares. O principal sistema de aquisição de manganês em fungos, no entanto, é constituído por transportadores da família Nramp. *C. gattii* apresenta apenas um membro dessa família de proteínas e é predito que se localize na membrana celular. A ausência desse transportador levou a um aumento da sensibilidade a agentes quelantes e a espécies reativas de oxigênio. Além disso, linhagens mutantes nulas para esse gene apresentaram redução da virulência em um modelo não mamífero de infecção. Sendo assim, nossos resultados demonstram a importância da manutenção da homeostase de manganês na levedura patogênica *C. gattii* e sua correlação com a virulência desse patógeno.

ABSTRACT

Cryptococcus gattii is one of the etiological agents of cryptococcosis, a disease that primarily affects the lungs and can spread to the central nervous system and other organs. Alveolar macrophages represent the host's first line of defense against respiratory pathogens, determining the outcome of the infection. Among the strategies utilized by the host to defend against invading microorganisms, nutritional immunity stands out, which consists of controlling the bioavailability of micronutrients at the site of infection. Manganese is an interesting sequestration target as it is essential for proper fungus function and infection progression. Although systems that regulate manganese homeostasis have been well studied in the model yeast *Saccharomyces cerevisiae,* little information is available regarding fungal pathogens and its association with virulence. In the present work, two manganese import systems in the pathogenic yeast *C. gattii* were functionally characterized. The *C. gattii ZIP3* gene is a direct ortholog of *ATX2* from *S. cerevisiae*, which encodes a transporter located to the Golgi apparatus and is responsible for the extrusion of manganese from this organelle into the cytoplasm. The absence of this gene product led to an increase in manganese tolerance, increased sensitivity to reactive oxygen species and alteration in expression of redox systems in the cell. Furthermore, cells lacking Zip3 presented a decrease in virulence in a non-mammalian model of infection, corroborating changes in classic virulence determinants in this yeast, such as melanin deposition, secretion of the polysaccharide glucuronoxylomannan and altered profile of extracellular vesicle secretion. The main manganese acquisition system in fungi, however, is constituted by transporters of the Nramp family. *C. gattii* has only one member of this protein family and is predicted to locate in the cell membrane. The absence of this transporter led to increased sensitivity to chelating agents and reactive oxygen species. Furthermore, null mutant strains for this gene presented reduced virulence in a non-mammalian model of infection. Therefore, our results demonstrate the importance of proper manganese homeostasis in the pathogenic yeast *C. gattii* and its correlation with virulence in this pathogen.

1. INTRODUÇÃO GERAL

1.1 *Cryptococcus gattii* e a criptococose

Embora sejam consideradas doenças negligenciadas pelos principais órgãos de saúde, doenças fúngicas apresentam uma alta incidência de 1 bilhão de pessoas ao redor do globo, com 1,5 milhões de mortes associadas estimadas (BONGOMIN *et al.*, 2017). Dentre essas, doenças fúngicas invasivas representam um grande desafio para sistemas de saúde devido a subnotificação de casos, desafio de diagnósticos precisos, dificuldade de tratamentos farmacológicos eficientes e surgimento de linhagens resistentes a antibióticos ao redor do mundo (VON LILIENFELD-TOAL *et al.*, 2019). Esse quadro se torna mais problemático em unidades de tratamento intensivo onde normalmente se encontram pacientes debilitados e/ou imunocomprometidos e a mortalidade associada a micoses sistêmicas é elevada.

A criptococose, também conhecida como doença de Busse-Buschke, é uma das principais micoses sistêmicas ao lado da candidíase sistêmica, pneumonia causada por *Pneumocystis jirovencii*, aspergilose invasiva e histoplasmose disseminada. Estimam-se, por ano, 278 mil casos em pacientes HIV positivos, com 223 mil desses casos evoluindo para a forma mais grave da doença, o quadro de meningite criptocócica. A doença possui maior incidência na África subsaariana e sudeste da Ásia e possui uma mortalidade estimada de 181 mil casos por ano, chegando a 80% de mortalidade dependendo da região. Ademais, mortes por criptococose constituem 15% das mortes totais anuais relacionadas a HIV/AIDS (RAJASINGHAM *et al.*, 2017).

As leveduras encapsuladas *Cryptococcus neoformans* e *Cryptococcus gattii* são os agentes etiológicos da criptococose, doença caracterizada por um quadro inicial pulmonar, comumente também afetando o sistema nervoso central (SNC). Embora ambas espécies pertençam ao mesmo gênero, elas diferem em relação a localização geográfica, hospedeiro alvo, progressão da infecção e manifestações clinicas (KWON-CHUNG *et al.*, 2014). *C. neoformans* possui uma distribuição

global e é considerado um patógeno oportunista, causando infecções em, majoritariamente, indivíduos imunocomprometidos. É o principal responsável pelos casos de meningite criptocócica visto que apresenta uma migração aumentada através da barreira hematoencefálica (LI, S. S. *et al.*, 2014). Já *C. gattii* é considerado um patógeno primário, podendo acometer tanto indivíduos imunocompetentes, como imunocomprometidos. É comumente associado ao quadro pulmonar da doença e formação de criptococomas, lesões sólidas com aspecto tumoral formados por substância gelatinosa, mas também é capaz de desenvolver infecção no SNC (GUSHIKEN; SAHARIA; BADDLEY, 2021; NGAMSKULRUNGROJ *et al.*, 2012). No ambiente, é isolado, principalmente, em madeira em decomposição e cavidades no tronco de árvores, em especial as do gênero *Eucalyptus,* classicamente em regiões de clima tropical e subtropical (GROVER *et al.*, 2007; HAGEN; BOEKHOUT, 2010).

Estima-se que *C. gattii* divergiu de *C. neoformans* há aproximadamente 37 milhões de anos e posteriormente nos sorotipos B e C, classificados de acordo com a composição estrutural da cápsula polissacarídica (BELAY *et al.*, 1996; LIN, X.; HEITMAN, 2006). Com o avanço das técnicas moleculares, como PCR *fingerprinting* e polimorfismo por tamanho de fragmento amplificado (AFLP), foi possível detectar subtipos moleculares que apresentam características fenotípicas distintas (Figura 1). Sendo as leveduras do sorotipo B associados aos tipos moleculares VGl e VGll e as do sorotipo C a VGlll e VGlV (LIN, X.; HEITMAN, 2006; SORRELL *et al.*, 1996).

Foi proposto, recentemente, que *C. neoformans* e *C. gattii* compreendem, na verdade, um complexo de espécies, e que fosse então feita uma separação por novos nomes (KWON-CHUNG *et al.*, 2017). *C. gattii* subtipo VGlla classicamente, ou *Cryptococcus deuterogattii* na nova nomenclatura, representa o maior interesse devido a algumas peculiaridades. Como a nova nomenclatura ainda é discutida e não amplamente aceita, usaremos a nomenclatura clássica ao longo do texto.

Linhagens do tipo VGlla de *C. gattii* foram responsáveis pelo surto que acometeu a ilha de Vancouver no Canadá e noroeste dos Estados Unidos a partir de 1999 (MA *et al.*, 2009; STEPHEN *et al.*, 2002). Enquanto outras linhagens de *C. gattii* são relacionadas a climas quentes, esse subtipo é, também, relacionado a

climas temperados, compreendendo casos ao redor do globo (BIELSKA; MAY, 2016). Durante os anos de 1999 a 2007, o surto afetou pelo menos 218 pessoas, levando a 19 óbitos (GALANIS *et al.*, 2010). A maioria dos pacientes sofreram de doença respiratória (77%), e/ou criptococomas pulmonares (75%) e um terço dos pacientes apresentaram também infecção no sistema nervoso central (GALANIS *et al.*, 2010; PHILLIPS *et al.*, 2015).

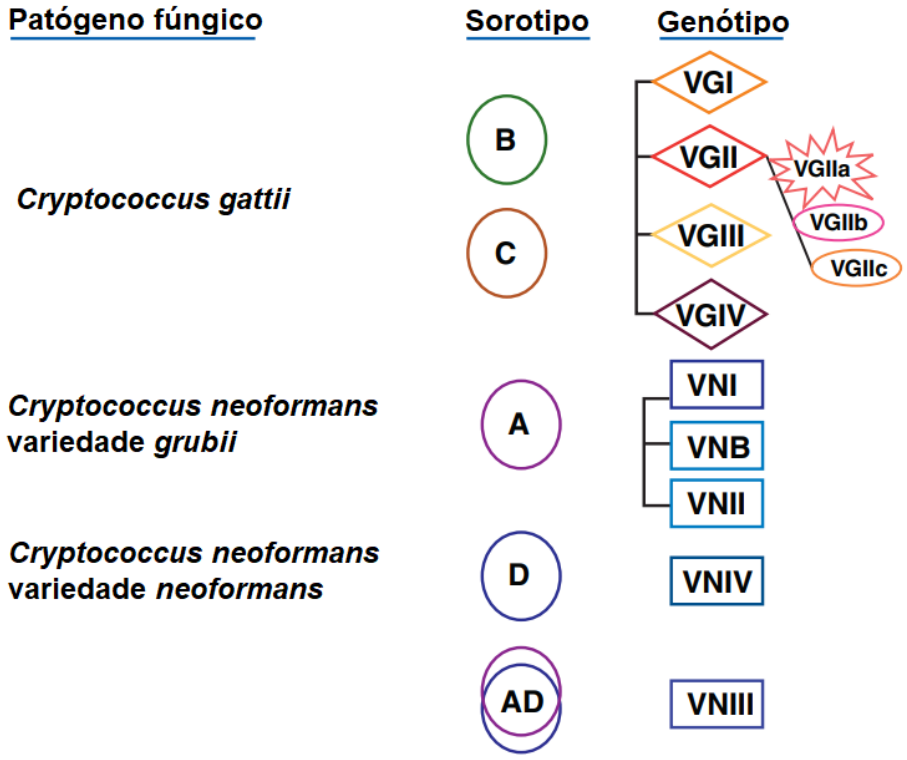

Figura 1: Sorotipos e subtipos moleculares de Cryptococcus spp. Linhagens de *C. gattii* pertencem aos sorotipos B ou C; linhagens pertencentes ao sorotipo BC são raramente reportadas. Linhagens de *C. neoformans var grubii* são sorotipo A, enquanto linhagens de *C. neoformans var neoformans* são sorotipo D; ainda existem linhagens hibridas AD. Subtipos moleculares de *C. gattii* e *C. neoformans* são baseados em tipagem de sequências multilocus de vários fragmentos de genes. Surtos de *C. gattii* na América do Norte são relacionados ao subtipo molecular VGIIa em destaque. Adaptado de (CHATURVEDI; CHATURVEDI, 2011).

O subtipo VGIIa apresenta diversas modificações comparadas a outras linhagens de *C. gattii* através de mutações e recombinações, rearranjos cromossômicos e, potencialmente, transferência gênica entre espécies do gênero *Cryptococcus* (D'SOUZA *et al.*, 2011; ENGELTHALER *et al.*, 2014). Curiosamente, foram perdidos 146 genes, um número três vezes maior que todos os outros subtipos moleculares de *C. gattii* juntos, incluindo genes que normalmente são

considerados essenciais, como a peroxidase mitocondrial citocromo c (CCP) (FARRER *et al.*, 2015). Por outro lado, a linhagem VGIIa também ganhou muitos genes codificantes únicos como citocromo c oxidase (COX6B), proteína de choque térmico 70 (HSP70) e proteínas possivelmente envolvidas na ligação de ferro e tráfico por membranas (FARRER *et al.*, 2015). Uma reversão numa mutação *frameshift* do gene de reparo ao DNA *msh2*, tornando-o funcional novamente, também foi encontrada e é possível que essa mudança contribua para a virulência aumentada dessa linhagem, visto que linhagens não patogênicas mantém a mutação (BLAKE BILLMYRE *et al.*, 2014).

Entre os genes perdidos pelo subtipo VGIIa, também se encontram os genes *Ago1* e *Ago2* que codificam a argonauta, proteína central na via clássica de RNA de interferência (D'SOUZA *et al.*, 2011). Essa ausência da maquinaria de RNA de interferência, que atua como mecanismo de defesa do genoma, pode levar a reativação de transposons, que podem, por sua vez, acelerar o processo de evolução do genoma e levar a novos mecanismos para lidar com as defesas do hospedeiro. O subtipo VGIIa é considerado o mais virulento e com a mais baixa susceptibilidade a drogas antifúngicas (TRILLES *et al.*, 2012).

Embora a maior parte dos casos de criptococose seja devido a infecções por *C. neoformans*, *C. gattii* tem ganhado maior notoriedade devido a sua capacidade de infectar indivíduos sadios e uma mudança de habitat para climas temperados, podendo ser também considerado um patógeno de distribuição global (AKINS; JIAN, 2019; CHATURVEDI; CHATURVEDI, 2011; DATTA *et al.*, 2009). Ainda, a doença pulmonar possui menores taxas de diagnóstico correto, pois pode ser confundida com câncer de pulmão, tuberculose, pneumonia bacteriana e outras micoses pulmonares tanto clinicamente quanto radiologicamente (SETIANINGRUM; RAUTEMAA-RICHARDSON; DENNING, 2019). De fato, estima-se que haja uma grande subnotificação de casos de infecção por *C. gattii* devido a diagnósticos incorretos (IVERSON *et al.*, 2012; TINTELNOT *et al.*, 2015). Apesar dos melhores tratamentos disponíveis, a mortalidade por infecções de *C. gattii* pode chegar até 33%, devido à demora de diagnóstico correto e dificuldades associadas ao tratamento de criptococomas (AKINS; JIAN, 2019; HARRIS *et al.*, 2011).

1.2 Via de infecção

Como dito anteriormente, as leveduras de *C. gattii* se encontram no ambiente e qualquer pessoa pode se infectar ao entrar em contato com o patógeno em áreas onde o fungo está presente, apesar de existirem alguns fatores predisponentes associados (SPRINGER; CHATURVEDI, 2010). Infecções por *C. gattii* não são contagiosas entre seres humanos e/ou animais. A infecção tem início por via respiratória (Figura 2), através da inalação de propágulos infecciosos, os quais são basidiósporos ou leveduras dessecadas, que apresentam um tamanho reduzido e, dessa forma, são capazes de atravessar as barreiras físicas do muco, ação ciliar e turbulência do ar e conseguem alojar-se nos alvéolos pulmonares (BARTEMES; KITA, 2018).

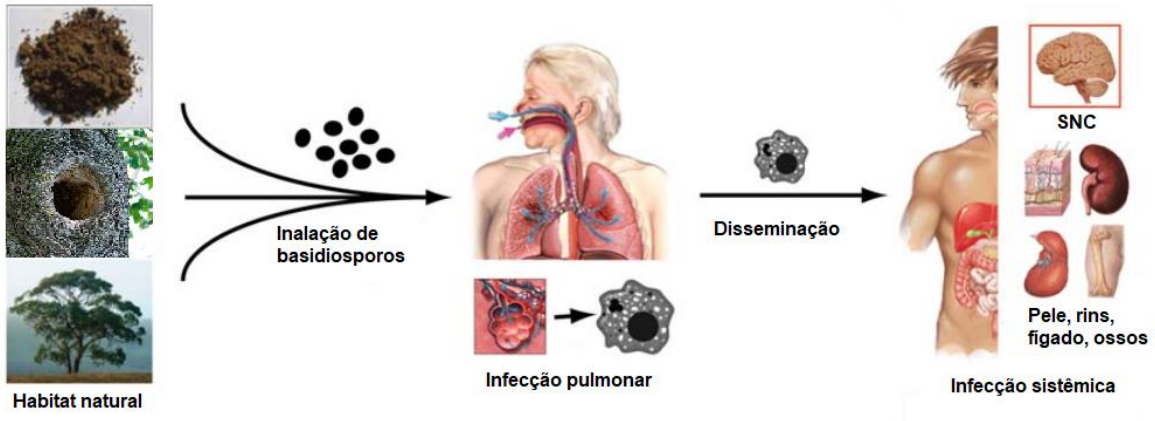

Figura 2: Esquema de infecção por C. gattii. O patógeno se encontra no ambiente, normalmente associado a árvores em decomposição ou do gênero *Eucalyptos.* A infecção ocorre a partir da inalação de esporos causando um quadro primário de infecção pulmonar. As leveduras patogênicas são então fagocitadas por macrófagos alveolares e podem tanto permanecer latentes no seu interior ou serem reativadas e se disseminar para o restante do corpo, incluindo órgãos como sistema nervoso central (SNC), pele, rins, fígado e ossos. Adaptado de (VOELZ, 2010).

Uma vez que essas partículas alcançam os pulmões, elas são desafiadas pelo sistema imune do hospedeiro, inicialmente na forma de fagócitos como os macrófagos alveolares, células dendríticas e os neutrófilos (OSTERHOLZER *et al.*, 2009b). Os macrófagos alveolares desempenham um papel central na contenção da infecção. Primeiramente, graças a estratégias de defesa como a fagocitose para eliminação de patógenos e liberação de citocinas pró-inflamatórias capazes de recrutar mais células imunes e, posteriormente, atuando como apresentadores de

antígeno para resposta imune adaptativa (MANSOUR *et al.*, 2014). As leveduras patogênicas podem também permanecer latentes no interior de macrófagos e serem reativadas posteriormente (HAGEN *et al.*, 2010, 2012).

Após o estabelecimento de uma infecção pulmonar primária, as leveduras podem se disseminar pelo organismo através da corrente sanguínea, podendo utilizar os próprios macrófagos do hospedeiro como veículo (CHEN, S. C.-A.; MEYER; SORRELL, 2014). Embora o alvo principal da infecção por *C. gattii* seja o pulmão, ele também é capaz de afetar outros órgãos como no SNC, pele, ossos, fígado, baço e os nódulos linfáticos (NGAMSKULRUNGROJ *et al.*, 2012; SABIITI; MAY, 2012).

1.3 Interações patógeno-hospedeiro

Diferentemente de *C. neoformans*, *C. gattii* é capaz de infectar indivíduos imunocompetentes, o que pode indicar que *C. gattii* utiliza métodos diferentes ou até adicionais para inibir a resposta imune do hospedeiro (BIELSKA; MAY, 2016). Ao contrário do que se esperaria, *in vitro*, *C. gattii* induz maiores concentrações de citocinas pro inflamatórias, como interleucina 1β (IL-1β), fator de necrose tumoral-α (TNF-α) e interleucina 6 (IL-6) em células humanas mononucleares de sangue periférico de humanos, além das citocinas de células T como interleucina 17 (IL-17) e interleucina 22 (IL-22) comparado a *C. neoformans* (SCHOFFELEN *et al.*, 2013). Sabe-se que os perfis de resposta Th1 e Th17 são protetores contra *Cryptococcus*, principalmente citocinas do perfil Th17 estão relacionadas a diminuição da carga fúngica pulmonar e um melhor prognóstico da doença (ZHANG *et al.*, 2009).

No entanto, em estudos em camundongos, *C. gattii* é capaz de modular a resposta imune do hospedeiro para longe do perfil Th1/Th17 atenuando a função de células dendríticas, as principais células envolvidas na apresentação de antígenos para células T (ANGKASEKWINAI *et al.*, 2014). Essa redução da atividade e maturação de células dendríticas também é relacionada a níveis reduzidos de TNF-α *in vivo* (HUSTON *et al.*, 2013). Recentemente, foi elucidado que *C. gattii* é capaz de impedir a fusão do fagossomo ao lisossomo e afetar a

funcionalidade de células dendríticas através da retenção de actina em uma estrutura protetora para si (JAMIL *et al.*, 2020). A infecção aguda induz um processo rápido de inflamação, porém uma resposta inflamatória sistêmica duradoura é impedida por inibição da maturação de células dendríticas e posterior ativação de células T. Esses dados podem ajudar a explicar por que há um menor crescimento de *C. gattii* em sangue quando comparado a *C. neoformans. In vitro* é montada uma resposta forte, pró-inflamatória contra *C. gattii*, o que não é observado no pulmão devido a atenuação das células dendríticas (NGAMSKULRUNGROJ *et al.*, 2012; SCHOFFELEN *et al.*, 2013). Ainda, células ou filtrados de cultura de *C. gattii* não são capazes de promover migração de leucócitos polimorfonucleares para o pulmão, e são capazes de afetar a migração e função de neutrófilos (CHENG; SHAM; KRONSTAD, 2009; WRIGHT *et al.*, 2002).

Os macrófagos podem, dependendo de moléculas sinalizadoras presentes no microambiente onde se encontram, ser ativados em um perfil clássico (M1), ou em um perfil alternativo (M2) (MURRAY *et al.*, 2014). Macrófagos M1 são ativados por citocinas pró-inflamatórias, como as do perfil Th1, e são associados a eliminação de patógenos, produção de espécies reativas de oxigênio (ROS), e produção de citocinas pró-inflamatórias (ATRI; GUERFALI; LAOUINI, 2018). A ativação alternativa, induzida por perfil de citocinas Th2, contribui para regulação da resposta inflamatória, fagocitose e limpeza de restos celulares e possui papel chave no processo de cicatrização, porém não é protetora contra patógenos (ATRI; GUERFALI; LAOUINI, 2018; LEOPOLD WAGER, C M; WORMLEY, 2014).

Na infecção, os macrófagos alveolares parecem desempenhar um papel paradoxal. Sabe-se da função fundamental dos macrófagos como primeira linha de defesa do hospedeiro, determinando o desfecho da infecção, esse papel é suportado por diversas evidências. No entanto, os macrófagos também podem ser utilizados como reservatórios e meios para disseminação pelo organismo. Depleção de macrófagos em diversos modelos animais de criptococose foram relacionados a uma maior carga fúngica (BOJARCZUK *et al.*, 2016; OSTERHOLZER *et al.*, 2009b; SHAO *et al.*, 2005). Além disso, recrutamento de monócitos, que podem então se diferenciar em macrófagos no tecido alvo, desempenha função fundamental na contenção da infecção (CHEN, G. H. *et al.*,

2007; OSTERHOLZER *et al.*, 2011; ZHANG *et al.*, 2010). Ainda, macrófagos ativados com perfil M1 são associados com melhores prognósticos em modelo de criptococose e em pacientes humanos (HARDISON *et al.*, 2010; JARVIS *et al.*, 2015). Por fim, macrófagos também parecem estar envolvidos na formação e/ou manutenção de criptococomas, que supõe-se que atue como barreira física para impedir a replicação e disseminação da infecção (FARNOUD *et al.*, 2015; MCQUISTON; LUBERTO; DEL POETA, 2010).

Para realizar seu papel na contenção da infecção, os macrófagos apresentam diversos mecanismos para eliminação de patógenos. As leveduras fagocitadas por macrófagos são engolfadas numa estrutura chamada fagossomo, que posteriormente sofre um processo de maturação ao se fundir com diversas classes de vesículas endocíticas e ao lisossomo formando o fagolisossomo, a organela especializada em lidar com patógenos. O fagolisossomo apresenta um ambiente hostil ao microrganismo fagocitado, caracterizado por um pH ácido, presença de espécies reativas de oxigênio e nitrogênio, escassez de nutrientes e presença de enzimas e peptídeos com ação antimicrobiana (GIBSON; JOHNSTON, 2015; UNDERHILL; GOODRIDGE, 2012). Além do seu papel ativo na eliminação de patógenos, macrófagos atuam na imunidade adaptativa como apresentadores de antígenos para células T e ainda secretando citocinas capazes de recrutar leucócitos para o sítio de infecção (LEOPOLD WAGER, C M; WORMLEY, 2014).

Embora, células de *Cryptococcus* sejam fagocitadas por macrófagos, existem diversos desfechos possíveis: (I) eliminação dos patógenos no fagolisossomo pelos mecanismos citados acima; (II) permanecer latentes no interior dos macrófagos; (III) replicação das leveduras no interior do macrófago e posterior lise do mesmo; (IV) transferência das células da levedura para outro macrófago sem causar lise; (V) escape do interior do macrófago sem causar a lise, um mecanismo conhecido como vomocitose (ALVAREZ; CASADEVALL, 2006; JOHNSTON; MAY, 2013). E mais vezes do que não, as células fúngicas são capazes de sobreviver e proliferar dentro dos macrófagos. Isolados considerados hipervirulentos estão relacionados a altas taxas de fagocitose por macrófagos e altas taxas de proliferação intracelular (HANSAKON *et al.*, 2019; MA *et al.*, 2009; MANSOUR *et al.*, 2011). Ainda, como discutido anteriormente, macrófagos podem ser utilizados como meio de

proliferação e disseminação pelas leveduras, inclusive para ultrapassar a barreira hematoencefálica e chegar ao SNC, num mecanismo conhecido como “Cavalo de Troia” (LIU, T.-B.; PERLIN; XUE, 2012).

1.4 Estratégias de virulência

Para sobreviver e proliferar no interior de macrófagos, as leveduras patogênicas apresentam um arsenal de estratégias de virulência que auxiliam na propagação da infecção. Acredita-se que muitas dessas estratégias surgiram após coevolução com predadores ambientais, como amebas de vida livre (STEENBERGEN; SHUMAN; CASADEVALL, 2001). Amebas são capazes de fagocitar bactérias e fungos no ambiente e utilizam estratégias semelhantes aos macrófagos para eliminação destes. Essa teoria é suportada por estudos comparando o perfil transcricional de leveduras fagocitadas por amebas e por macrófagos murinos (DERENGOWSKI *et al.*, 2013). Curiosamente, *C. gattii* é menos fagocitado pelo modelo ameba *Acanthamoeba castellanii* do que *C. neoformans*, talvez refletindo diferenças na estrutura de suas cápsulas (MALLIARIS; STEENBERGEN; CASADEVALL, 2004).

As espécies do complexo *Cryptococcus* são conhecidas pela produção de uma cápsula polissacarídica, uma estrutura altamente hidratada e negativamente carregada, que auxilia no diagnóstico da doença. A cápsula varia de tamanho dependendo de diversos estímulos, mas sabe-se que é induzida em meios estressores, que mimetizam o hospedeiro e *in vivo* (GARCIA-HERMOSO; DROMER; JANBON, 2004). A cápsula é composta, principalmente, por glicuronoxilomanana (GXM), composto por manose, xilose e ácido glicurônico, responsável por cerca de 95% da massa capsular (ZARAGOZA *et al.*, 2009). E em menor proporção por galactoxilomanana (GalXM) e manoproteínas associadas (VARTIVARIAN *et al.*, 1989). Os componentes são sintetizados internamente e secretados para o meio extracelular via vesículas, a montagem da cápsula ocorre, provavelmente, de fora para dentro (NIMRICHTER *et al.*, 2007). Ademais, sabe-se

que a agregação de GXM capsular é dependente de cátions divalentes (RODRIGUES *et al.*, 2007).

A cápsula polissacarídica possui diversas funções na patogenicidade dessas leveduras. Na ausência de opsoninas, ela é capaz de inibir a fagocitose das leveduras por macrófagos, agindo, também, como uma proteção ao encobrir padrões moleculares associados a patógenos (PAMPs), como moléculas presentes na parede celular fúngica (KOZEL, 1993; LEOPOLD WAGER, Chrissy M *et al.*, 2016). A cápsula também auxilia a escapar de neutrófilos através da modulação da produção de armadilhas extracelulares de neutrófilos (NETs) (ROCHA *et al.*, 2015). Além disso, é descrita a capacidade da cápsula em proteger a levedura de ROS e peptídeos antimicrobianos, promovendo a sobrevivência no fagolisossomo (ZARAGOZA *et al.*, 2008).

Os açucares que compõem a cápsula são capazes de, por si só, modelarem o sistema imune de mamíferos. Correlacionados a supressão de uma resposta inflamatória sistêmica, devido a supressão da proliferação de células T, indução da secreção da citocina anti-inflamatória interleucina 10 (IL-10) e inibição da secreção de TNF-α e IL-1β por monócitos humanos (SYME *et al.*, 1999; VECCHIARELLI, A. *et al.*, 1995; VECCHIARELLI, Anna *et al.*, 1996). Além disso, essas moléculas são capazes de interferir na migração de leucócitos, na secreção da citocina pró-inflamatória interleucina 17A (IL-17A) por células T, na apresentação de antígenos via complexo principal de histocompatibilidade (MHC) e induzir apoptose em linfócitos e macrófagos (ELLERBROEK *et al.*, 2004; PERICOLINI *et al.*, 2013; VECCHIARELLI, Anna *et al.*, 2011). Curiosamente, GXM de baixa massa molecular purificado de *C. gattii* foi capaz de induzir a produção de óxido nítrico (NO) e ativar a via *toll-like receptor 2* (TLR-2) e *factor nuclear kappa β* (NF-Kβ) em macrófagos (FONSECA *et al.*, 2010). Produção aumentada de NO também foi observada após exposição de macrófagos a vesículas extracelulares (EVs), que foi diminuída após a adição de GXM, sugerindo que esses dois componentes atuem juntos para reduzir a resposta pró-inflamatória do hospedeiro (OLIVEIRA, D. L. *et al.*, 2010).

Outra característica considerada essencial para patógenos humanos é a capacidade de se desenvolver a temperatura elevadas. De fato, esse pode ser considerado o maior diferencial entre espécies patogênicas e não patogênicas de

Cryptococcus (PETTER *et al.*, 2001). Isso é possível através da via regulada por calcineurina, uma fosfatase serina/treonina sensível a cálcio (LIU, J. *et al.*, 1991). Normalmente, linhagens que não possuem esse gene são consideradas avirulentas em *C. gattii* e *C. neoformans,* devido a incapacidade de desenvolvimento a temperatura do hospedeiro. No entanto, linhagens de *C. gattii* VGlla que não apresentam calcineurina funcional são viáveis em altas temperaturas, embora apresentem uma severa atenuação da virulência (CHEN, Y. L. *et al.*, 2013).

A atividade da enzima lacase constitui outro determinante de virulência clássico de *Cryptococcus*. Lacases são oxidases dependentes de cobre presentes em diversas plantas, fungos e bactérias. Em *Cryptococcus,* a atividade de lacase está relacionada a diversos outros mecanismos que auxiliam na virulência, como produção de melanina, fosfolipase B (Plb1), urease e superóxido dismutase (SOD1) (CHEN, S. C.-A.; MEYER; SORRELL, 2014). A partir de compostos fenólicos, como L-DOPA, é produzido o pigmento melanina, que já foi descrito como capaz de estabilizar radicais livres, atuando como antioxidante contra ROS gerados pelo hospedeiro (WANG, Y.; CASADEVALL, 1994). O pigmento também parece ser importante na proteção contra fagocitose, morte por leucócitos do hospedeiro, e peptídeos antimicrobianos (KWON-CHUNG *et al.*, 2014). Além disso, o pigmento também é capaz de absorver radiação eletromagnética, protegendo o organismo de vida livre e é um importante constituinte da parede celular, aumentando sua rigidez (DADACHOVA *et al.*, 2008; WILLIAMSON, 1997). A agregação de grânulos de melanina também é promovida por cátions divalentes (CAMACHO *et al.*, 2019). Por fim, a melanina é, também, capaz de proteger o fungo de diversos agentes antifúngicos como anfotericina B, caspofungina e azóis (KWON-CHUNG *et al.*, 2014).

Dentre as enzimas secretadas pelas leveduras, destacam-se a urease e a fostolipase B. A urease catalisa a hidrólise de ureia a amônia, e em *Cryptococcus* parece estar envolvida com a transmigração e invasão do SNC (OLSZEWSKI *et al.*, 2004). Também é importante para a propagação da infecção no pulmão, já que auxilia na sobrevivência intracelular em macrófagos e promove a acumulação de células dendríticas imaturas, assim como uma resposta Th2 não protetiva (FEDER *et al.*, 2015; OSTERHOLZER *et al.*, 2009a). Já as fosfolipases atuam na hidrólise

de ligações de fosfolipídios, como os presentes nas membranas das células do hospedeiro. A secreção de Plb1 está relacionada com a sobrevivência das leveduras fagocitadas por macrófagos, e é teorizado que atue na permeabilização do fagolisossomo, dessa forma tendo papel direto na obtenção de nutrientes (CHRISMAN *et al.*, 2011; COX *et al.*, 2001).

Altas taxas de replicação intracelular em linhagens de *C. gattii* do surto do noroeste do pacífico são associadas a um mecanismo único, alterações na morfologia mitocondrial, as mitocôndrias passam a apresentar uma forma tubular. (MA *et al.*, 2009; VOELZ *et al.*, 2014). Inicialmente, hipotetizou-se que essa alteração mitocondrial estava relacionada a proteção contra o ambiente hostil do fagolisossomo, principalmente a espécies reativas de oxigênio geradas pelo hospedeiro, mas na verdade fazia parte de um modelo mais complexo. Após entrada nos fagócitos, células de *C. gattii* ativam um modelo de "divisão de trabalho", onde algumas células adotam essa modificação mitocondrial e deixam de se dividir, porém, ao fazerem isso, elas possibilitam a rápida proliferação das células vizinhas ao neutralizar espécies reativas de oxigênio produzidos pelos fagócitos, dessa forma, amplificando a proliferação da população como um todo (VOELZ *et al.*, 2014).

Vesículas extracelulares de *Cryptococcus* participam na exportação de fatores de virulência que impactam profundamente a interação patógeno-hospedeiro. Dentre os conteúdos presentes nessas vesículas está o principal polissacarídeo da cápsula GXM, além de proteínas importantes na virulência, como a lacase e a urease (RODRIGUES *et al.*, 2008, 2007). As vesículas atuam como veículo de comunicação entre as células patogênicas a longa distância, sendo responsáveis por mediar o processo de "divisão de trabalho" citado acima, inclusive para aumentar a proliferação intracelular de linhagens não-virulentas (BIELSKA *et al.*, 2018). As vesículas também são rapidamente captadas pelas células do hospedeiro, e, dessa forma, conseguem impactar em processos como fagocitose e secreção de citocinas (DE OLIVEIRA, H. C. *et al.*, 2020).

Sabe-se que o aumento do diâmetro da cápsula polisssacarídica está relacionado a proteção contra a fagocitose e estresse oxidativo realizado pelos fagócitos do hospedeiro (ZARAGOZA *et al.*, 2008). No entanto, *in vivo*, pode haver

aumento do tamanho do corpo celular também, formando células gigantes ou titãs (OKAGAKI *et al.*, 2010; ZARAGOZA *et al.*, 2010). Essas células apresentam entre 50 e 100 µm de diâmetro em comparação aos 4 a 10 µm normais, e representam cerca de 20% da população de *Cryptococcus* durante a infecção pulmonar. Na presença dessas células titãs, o processo de fagocitose é diminuído, não só para as células gigantes (OKAGAKI; NIELSEN, 2012).

1.5 Homeostase de manganês

Metais de transição como ferro, zinco e manganês são indispensáveis para a vida. Dentre os diversos papéis em processos biológicos, metais de transição são mais conhecidos por participar do metabolismo central e equilíbrio redox (GERWIEN *et al.*, 2018). Devido as suas propriedades únicas, íons desses metais são comumente incorporados em metaloproteínas, incluindo enzimas, proteínas de armazenagem de metais e fatores de transcrição. Metais se tornaram tão predominantes em processos biológicos que mais de 30% de todas as proteínas são preditas como capazes de utilizar um cofator metálico, estando envolvidos em virtualmente metade de todas as reações enzimáticas (ANDREINI *et al.*, 2008). No entanto, sua alta reatividade, que é tão utilizada para catálise, pode tornar esses metais tóxicos em altas concentrações. Dessa maneira, células precisam regular finamente os níveis intracelulares de metais para proliferarem, enquanto evitam potenciais efeitos danosos (ROBINSON; ISIKHUEMHEN; ANIKE, 2021).

Íons de manganês são necessários em diversos processos biológicos, destacando-se no controle do estresse oxidativo. Esse metal redox ativo é um cofator essencial, atuando tanto no sítio catalítico como estruturalmente, para enzimas envolvidas em diversos papeis fundamentais, como biossíntese de desoxinucleotídeos (ribonucleotídeo redutase), metabolismo de carbono (fosfoglicerato mutase), fosforilação (serina/treonina quinase), estresse oxidativo (superóxido dismutase 2), glicosilação no Golgi (transferases de açúcar), assim como polimerases de RNA e DNA, dentre outras (CROWLEY; TRAYNOR; WEATHERBURN, 2000; KELLIHER; KEHL-FIE, 2016). Diversos patógenos

fúngicos, incluindo *C. neoformans* e *C. gattii*, expressam uma isoforma de superóxido dismutase dependente de manganês (SOD2) como estratégia contra ROS gerados pelo hospedeiro (FRÉALLE *et al.*, 2005). Além disso, manganês é único no sentido de que apresenta atividade antioxidante mesmo em altas concentrações, em contraste com outros metais de transição que apresentam função anti e pró-oxidantes. Complexos não proteicos contendo manganês agem na neutralização de ROS, especialmente complexos contendo lactato ou fosfato (BARNESE *et al.*, 2008, 2012).

Como dito anteriormente, a regulação dos níveis de íons metálicos é fundamental para o correto funcionamento celular. Para manter um controle homeostático sobre os níveis de manganês, as células expressam uma variedade de transportadores que participam no transporte especifico de manganês ou no transporte geral de cátions divalentes (CULOTTA; YANG; HALL, 2005; DISS *et al.*, 2011). Os mecanismos associados a mobilização intracelular de manganês foram melhor estudados no modelo *Saccharomyces cerevisiae* (Figura 3), enquanto há menos informação disponível para patógenos fúngicos.

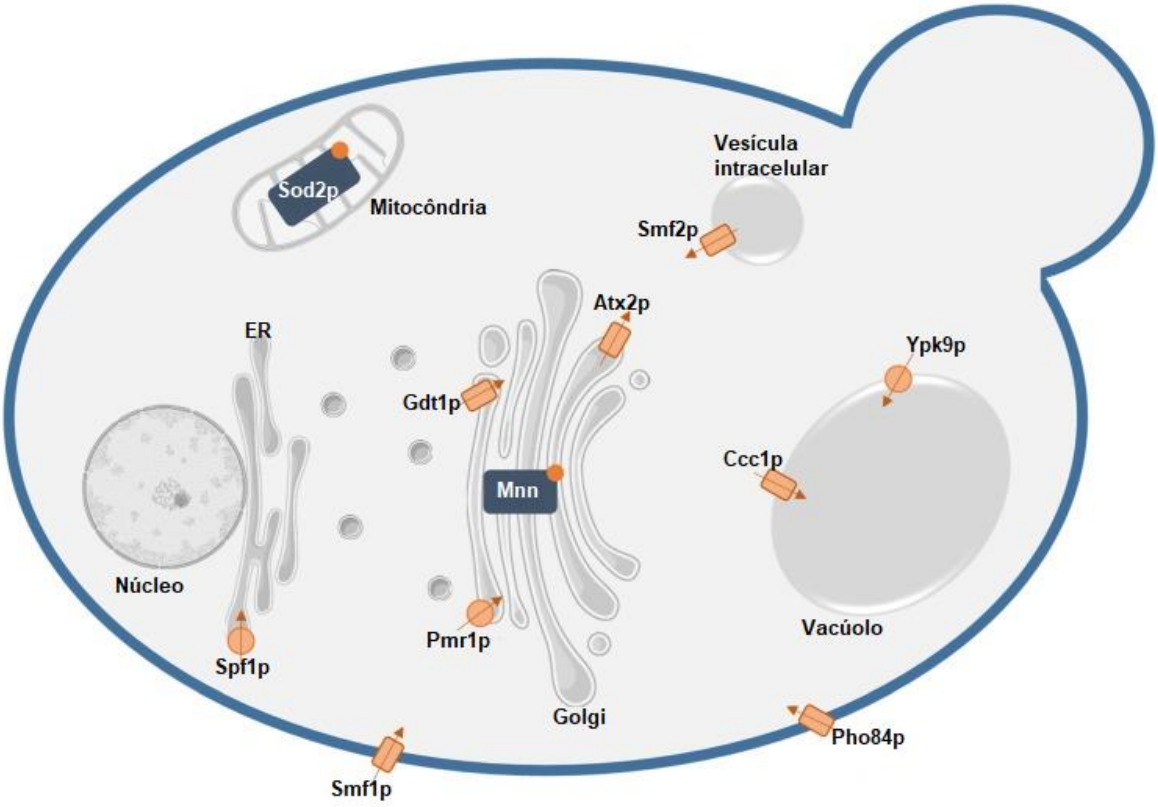

Figura 3:Vias de transporte de manganês sugeridas na levedura Saccharomyces cerevisiae. Os transportadores identificados até o momento envolvidos no transporte de Mn^{+2} são: Smf1p, um transportadores da família *Natural Resistance-Associated Macrophage Protein* (NRAMP) presente na membrana plasmática; Pho84p, um transportador de fosfato de alta afinidade capaz de transportar Mn^{+2}; Pmr1p, um transportador P-ATPase presente no Golgi capaz de transportar Ca^{+2} e Mn^{+2}; Gdt1p, um transportador secundário presente no Golgi; Atx2p, um transportador de Mn^{+2} presente no Golgi; Spf1p, um transportador P-ATPase presente no retículo endoplasmático (ER); Smf2p, um transportador NRAMP localizado em vesículas; Ypk9p, um transportador P-ATPase que importa Mn^{+2} pro vacúolo; Ccc1p, um transportador vacuolar de Fe^{+2} e Mn^{+2}; Esses transportadores garantem manutenção dos níveis intracelulares de manganês dentro do limite fisiológico e para atividade de enzimas que utilizam Mn^{+2}: a superóxido dismutase Sod2p na matriz mitocondrial e transferases de açúcar (Mnn) no lúmen do Golgi. As flechas representam o sentido do transporte de Mn^{+2}, enquanto retângulos e círculos correspondem a transportadores secundários e ATPases, respectivamente. Adaptado de (THINES *et al.*, 2019).

A aquisição de manganês por células fúngicas é realizada, principalmente, por proteínas da família Nramp (*Natural resistance-associated marophage protein*), uma classe de transportadores de metais conservada de bactéria a mamíferos (CELLIER, M. *et al.*, 1995). Transportadores Nramp são responsáveis pela translocação de uma série de metais divalentes através de membranas para o citoplasma, utilizando um gradiente de prótons, incluindo manganês, ferro, cobre, cádmio e cobalto (PORTNOY; LIU; CULOTTA, 2000). Na levedura modelo *S.*

cerevisiae, Smf1p é responsável pela aquisição extracelular de manganês, estando presente na membrana plasmática em condições de escassez de manganês e sendo considerado um transportador de alta afinidade (LIU, X. F.; CULOTTA, 1999; SUPEK *et al.*, 1996). Linhagens nulas para *SMF1* não apresentaram alteração nos níveis intracelulares de manganês comparadas a linhagem selvagem, além de nenhum defeito observável na atividade de enzimas que utilizam manganês (LUK, E. E. C.; CULOTTA, 2001; SUPEK *et al.*, 1996). No entanto, Smf1p parece ser importante na resistência ao estresse oxidativo, fornecendo manganês para complexos antioxidantes não proteicos, visto que a adição de sais de manganês a células sem uma defesa antioxidante funcional reverte todos os fenótipos observados, e esse resgate é dependente de Smf1p, mas não do parálogo Smf2p (LUK, E. E. C.; CULOTTA, 2001).

Em contraste, Smf2p reside em vesículas intracelulares derivadas do Golgi e participa da manutenção da homeostase de manganês em condições de níveis fisiológicos do mesmo, atuando como fornecedor de manganês armazenado para uso intracelular (PORTNOY; LIU; CULOTTA, 2000). O manganês mobilizado por Smf2p é biodisponível e utilizado por enzimas intracelulares, como a Sod2p (LUK, E. E. C.; CULOTTA, 2001). Um terceiro transportador Nramp, Smf3p, também está presente, no entanto, está envolvido na translocação de, principalmente, ferro de vacúolos (COHEN, A.; NELSON; NELSON, 2000).

Em *S. cerevisiae*, a regulação da expressão dos transportadores Nramp Smf1p e Smf2p não ocorre a nível transcricional, mas sim num nível pós-traducional por reciclagem de proteína e mudança de localização em resposta a concentrações de manganês (JENSEN *et al.*, 2009; LIU, X. F.; CULOTTA, 1999; PORTNOY; LIU; CULOTTA, 2000). Esses transportadores são expressos constitutivamente, e nenhuma alteração em níveis de mRNA foi observada durante escassez de manganês. Em condições de manganês suficientes, os transportadores são endereçados para degradação em vacúolos. Enquanto que, em condições de escassez, a quantidade de transportadores Nramp aumenta rapidamente visto que Smf1p é endereçado para a membrana celular e Smf2p para vesículas intracelulares. A mudança de localização é mediada pela proteína associada a via

exocitica Bsd2p e a ligase de ubiquitina Rsp5p (JENSEN *et al.*, 2009; LIU, X. F.; CULOTTA, 1999; PORTNOY; LIU; CULOTTA, 2000).

Além dos transportadores Nramp de alta afinidade, outras proteínas transmembrana estão envolvidas na obtenção de manganês. Em altas concentrações do metal, o principal transportador envolvido na aquisição de manganês em leveduras é Pho84p, um membro da família MFS (*Major facilitator family*) (JENSEN; AJUA-ALEMANJI; CULOTTA, 2003). Pho84p é um transportador simporte de fosfato/próton com alta afinidade por fosfato, desempenhando um papel chave na obtenção de manganês extracelular em altas concentrações do mesmo, quando os transportadores Nramp não estão funcionais (JENSEN; AJUA-ALEMANJI; CULOTTA, 2003). Células sem Pho84p são mais tolerantes a concentrações tóxicas de manganês devido a menor acumulação do metal. Entretanto, essas linhagens não exibem qualquer defeito na atividade de enzimas que utilizam manganês ou em seu balanço redox (JENSEN; AJUA-ALEMANJI; CULOTTA, 2003; REDDI *et al.*, 2009).

Manganês também pode ser obtido de estoques intracelulares pela atividade do transportador Atx2p, da família ZIP (*Zrt/Irt-like protein*). Transportadores da família ZIP estão presentes em todos os domínios da vida e estão envolvidos no transporte de, principalmente, zinco para o citoplasma (GUERINOT, 2000). No entanto, a proteína Atx2p de *S. cerevisiae* se localiza na membrana de um compartimento remanescente do Golgi e é responsável pelo transporte de manganês (LIN, S. J.; CULOTTA, 1996). A superexpressão de Atx2p resulta em acúmulo de manganês no citoplasma da célula e é capaz de suprimir dano oxidativo causado por fontes externas.

O exporte de manganês do citoplasma é realizado por transportadores específicos para dentro de organelas ou para o espaço extracelular. A proteína Mtm1p, da família MC (*mitochondrial carrier*), foi isolada como possível responsável pelo transporte de manganês através da membrana interna da mitocôndria para que a Sod2p pudesse funcionar (LUK, E. *et al.*, 2003). No entanto, a proteína parece estar envolvida com a função da Sod2p de outra maneira, sendo, na verdade, envolvida no transporte de piroxidal 5-fosfato (PLP), a forma ativa da vitamina B_6 (WHITTAKER; PENMATSA; WHITTAKER, 2015; WHITTAKER; WHITTAKER,

2014). Dessa forma, ainda não se sabe o transportador responsável pela translocação de manganês para a matriz mitocondrial.

Em relação ao retículo endoplasmático, a mobilização de manganês para o lúmen da organela ocorre em função de Spf1p, também conhecido como Cod1p, um membro da família *P5A-type ATPase*. Linhagens ausentes em Spf1p apresentam redução no conteúdo luminal de manganês e processos relacionados a manganês no retículo, além de um aumento nos níveis e processos de manganês no citoplasma (COHEN, Y. *et al.*, 2013). A ausência de Spf1p também está relacionada a um fenótipo severo de estresse no retículo, com o fenótipo oposto sendo observado na linhagem de superexpressão (COHEN, Y. *et al.*, 2013). Spf1p também parece estar envolvido na homeostase de esteróis, pois linhagens defectivas nessa proteína apresentaram maior sensibilidade a inibidores da produção de esteróis, acumulo de esteróis na membrana plasmática e de corpos lipídicos no citoplasma (SØRENSEN *et al.*, 2019). Além de desempenhar um papel, junto aos transportadores do Golgi Pmr1p e Gdt1p, no correto processamento de glicoproteínas (VASHIST *et al.*, 2002).

Em concentrações suficientes de manganês, exporte do metal do citoplasma pode ser necessário para evitar toxicidade. Sequestro do metal em vacúolos é realizado pelo transportador Ccc1p da família VIT (*vacuolar iron transporter*) como forma de manter níveis homeostáticos de manganês (LAPINSKAS, Paula Jean; LIN; CULOTTA, 1996; LI, L. *et al.*, 2001). Homólogos de Ccc1 são bem distribuídos pela árvore da vida com exceção de Metazoa, e também estão envolvidos no metabolismo de ferro e cálcio em fungos (LI, L. *et al.*, 2001; SORRIBES-DAUDEN *et al.*, 2020). Consistente com um papel no sequestro de manganês, linhagens ausentes no transportador apresentaram maior sensibilidade a manganês, e a superexpressão de Ccc1p foi capaz de reduzir a toxicidade associada ao metal em linhagens defectivas em Pmr1p (LAPINSKAS, Paula Jean; LIN; CULOTTA, 1996). Ainda não foi identificado um transportador envolvido no efluxo de manganês do vacúolo, dessa forma, supõe-se que Ccc1p atue sequestrando manganês e não armazenando-o. Entretanto, transportadores vacuolares associados com outros metais podem facilitar a saída de manganês da organela em certas condições.

Ainda sobre transporte vacuolar, o sequestro de manganês também pode ser realizado, possivelmente, pelo transportador Cos16p (PAIDHUNGAT; GARRETT, 1998). O transportador Ypk9p também está localizado na membrana vacuolar e desloca manganês para o interior do vacúolo, como demonstrado pela hipersensibilidade a manganês observada em linhagens ausentes dessa proteína (GITLER *et al.*, 2009; SCHMIDT *et al.*, 2009). E uma quarta proteína que afeta a toxicidade por manganês presente na membrana do vacúolo é Mam3p, um membro da família ACDP (*ancient conserved domain protein*) (WANG, C. Y. *et al.*, 2003). Contrário aos demais transportadores vacuolares, que auxiliam na detoxificação de manganês, Mam3p parece aumentar a toxicidade pelo metal, visto que células ausentes em Mam3p eram mais tolerantes a concentrações tóxicas e a sua superexpressão diminuía tal tolerância (YANG *et al.*, 2005).

O transportador Pmr1p, da superfamília *P-type ATPase*, desempenha dois papéis fundamentais associados a homeostase de manganês, embora esteja primariamente envolvido no transporte de cálcio (ANTEBI; FINK, 1992). Primeiro, Pmr1p localiza na membrana do Golgi e atua como um fornecedor de manganês para enzimas presentes no lúmen do Golgi, como transferases de açúcar, dessa forma, permitindo o processamento e tráfego de proteínas através da via exocítica (ANTEBI; FINK, 1992; DÜRR *et al.*, 1998). Na ausência de Pmr1p, o direcionamento e glicosilação de proteínas no Golgi é prejudicado (DÜRR *et al.*, 1998; RUDOLPH *et al.*, 1989). Segundo, atuando como principal rota para eliminação de manganês tóxico de dentro da célula através da via exocítica, visto que células defectivas nesse transportador apresentam acúmulo do metal no citosol e hipersensibilidade a manganês (LAPINSKAS, P J *et al.*, 1995).

De maneira similar, Gdt1p é um trocador Ca^{2+}/H^{+} presente no Golgi, da família de proteínas transmembrana UPF0016, que também está envolvido na mobilização de manganês (THINES *et al.*, 2018). Gdt1p também é responsável por suprir as transferases de açúcar no lúmen do Golgi com manganês (DULARY *et al.*, 2018). E ainda, está envolvido na resistência a concentrações tóxicas de manganês e controle do armazenamento do metal. A ausência do transportador ainda foi relacionada a diminuição da atividade de Sod2p (THINES *et al.*, 2018). Uma terceira proteína parece participar no exporte e detoxificação de manganês na célula, a

permease de alta afinidade por histidina Hip1p, visto que células portando uma certa mutação nesse transportador, *hip1-272*, apresentaram menores níveis intracelulares de manganês devido ao aumento da atividade de efluxo, assim como apresentaram maior tolerância ao metal (FARCASANU *et al.*, 1998).

1.6 Imunidade nutricional

O sistema imune do hospedeiro comumente controla a biodisponibilidade de metais de transição no sítio de infecção como estratégia contra patógenos. Essa estratégia, chamada de imunidade nutricional, já foi descrita para diversos patógenos, e pode afetar as células invasoras tanto por remover nutrientes do seu alcance ou utilizar a toxicidade proveniente desses metais contra os patógenos (HOOD; SKAAR, 2012). Apesar da maior quantidade de informação a respeito do sequestro de ferro, outros metais de transição também são regulados no sítio de infecção, como zinco e manganês (KEHL-FIE; SKAAR, 2010). Por outro lado, os patógenos também utilizam diversas estratégias para lidar com essa ausência de metais, por exemplo, expressando transportadores de metais de alta afinidade ou enzimas capazes de usar mais de um metal como cofator (BARWINSKA-SENDRA *et al.*, 2020; GARCIA *et al.*, 2017).

O hospedeiro é capaz de restringir manganês de patógenos tanto intracelularmente quando extracelularmente. Patógenos intracelulares enfrentam um ambiente pobre em manganês devido a atividade do transportador de cátions divalentes NRAMP1 (SLC11A1), também conhecido como DMT-2. Esse transportador é constitutivamente expresso em macrófagos, neutrófilos e linfócitos e se localiza na membrana de lisossomos e fagossomos maturados onde age na retirada de, principalmente, ferro e manganês do fagolisossomo em direção ao citoplasma (GRUENHEID *et al.*, 1997; HEDGES *et al.*, 2013; JABADO *et al.*, 2000). Perda do transportador NRAMP1 em modelo murino resultou na maior susceptibilidade a uma variedade de patógenos intracelulares como *Candida albicans, Salmonella enterica, Mycobacterium lepraemurium, Leishmania donovani* e mais (BELLAMY, 1999; PULITI *et al.*, 1995; VIDAL *et al.*, 1995). Em humanos,

polimorfismos na região codificadora do gene *NRAMP1* foram relacionados ao aumento do risco de desenvolver diversas doenças relacionadas a patógenos intracelulares como tuberculose, leishmaniose e mais (CELLIER, M. F.; COURVILLE; CAMPION, 2007; LI, X. W. *et al.*, 2011). Ainda, NRAMP1 apresenta uma função sinérgica com outros mecanismos de defesa como síntese de espécies reativas de oxigênio e nitrogênio e modulação do perfil de expressão de citocinas de células imunes (FRITSCHE *et al.*, 2008; VALDEZ *et al.*, 2008).

Os níveis de manganês extracelular também são regulados pelo hospedeiro durante a infecção. O heterodímero S100A8/S100A9 calprotectina atua como mecanismo de defesa contra patógenos e é capaz de ligar zinco e manganês com alta afinidade (BROPHY; NOLAN, 2015; DAMO *et al.*, 2013). Calprotectina é um membro da família de proteínas ligantes de cálcio *EF-hand* e é secretada, principalmente, por neutrófilos, onde constitui quase metade de todo conteúdo proteico do citoplasma dessas células, mas também pode ser secretado por células epiteliais após estimulação (DALE; FAGERHOL; NAESGAARD, 1983; ZINDL *et al.*, 2013). Camundongos deficientes em produzir calprotectina apresentam menor capacidade de sequestrar manganês e são mais suscetíveis a uma variedade de infecções, incluindo fungos como *C. albicans, Aspergillus fumigatus e Aspergillus nidulans* (BIANCHI *et al.*, 2011; CLARK *et al.*, 2016; URBAN *et al.*, 2009). O efeito quelante da calprotectina também foi demonstrado *in vitro*, como capaz de reduzir o crescimento de patógenos fúngicos como *C. albicans, A. nidulans, A. fumigatus e C. neoformans* (BIANCHI *et al.*, 2011; CLARK *et al.*, 2016; MAMBULA *et al.*, 2000; URBAN *et al.*, 2009).

1.7 Imunidade nutricional relacionada a manganês e patógenos fúngicos

Para as células fúngicas, a privação de manganês pode resultar em efeitos severos. Como mencionado anteriormente, células fúngicas dependem de manganês para uma variedade de processos. Por exemplo, um dos principais sistemas para combater o estresse oxidativo é a isoforma mitocondrial da superóxido dismutase (SOD2), que é dependente de manganês. Células

deficientes em atividade de SOD2 normalmente apresentam fenótipos severos associados a altas temperaturas e estresse oxidativo, e profundo impacto na virulência, incluindo *C. neoformans e C. gattii* (NARASIPURA *et al.*, 2005; NARASIPURA; CHATURVEDI; CHATURVEDI, 2005). Outro exemplo seriam os defeitos observados na glicosilação e montagem da parede celular observados em *C. albicans, Candida guilliermondii* e *A. nidulans* devido à ausência do transportador de manganês PMR1, responsável por fornecer o metal para as transferases de açúcar do Golgi (BATES *et al.*, 2005; JIANG *et al.*, 2014; NAVARRO-ARIAS *et al.*, 2016).

Embora o papel do manganês seja bem entendido na defesa do hospedeiro a bactérias, pouco se sabe sobre a sua função contra patógenos fúngicos (KEHL-FIE; SKAAR, 2010; KELLIHER; KEHL-FIE, 2016; MOREY; MCDEVITT; KEHL-FIE, 2015; WU *et al.*, 2021). Como o hospedeiro apresenta a capacidade de restringir patógenos fúngicos de manganês, e a perturbação da homeostase desse metal em patógenos fúngicos afeta diversos processos celulares, incluindo a virulência, pode-se assumir que o hospedeiro utiliza estratégias similares as usadas contra outros patógenos para defender contra patógenos fúngicos.

Informação a respeito do controle da homeostase de manganês em patógenos fúngicos também é limitada. Sabe-se que transportadores homólogos aos Nramp de *S. cerevisiae* estão presentes na maioria dos genomas fúngicos, com exceção dos patógenos de planta *Magnaporthe grisea* e *Alternaria brassiccola* (DISS *et al.*, 2011). No entanto, o número de cópias desses genes relacionados a transportadores Nramp varia entre um e cinco por genoma. Em *C. neoformans*, existe apenas uma cópia e foi caracterizado como um transportador dependente de pH capaz de transportar manganês, ferro, cobalto e níquel se localizando na membrana plasmática (AGRANOFF *et al.*, 2005). *C. gattii* apresenta apenas uma cópia de genes relacionados a transportadores Nramp também.

Já sobre o transportador da família ZIP presente no Golgi, homólogos de *ATX2* de *S. cerevisiae* são bem conservados pelos genomas fúngicos, normalmente como cópia única, tendo como exceção os membros da classe Schizosaccharomycete que não apresentam nenhuma cópia (DISS *et al.*, 2011). Nosso grupo caracterizou os transportadores Zip1 e Zip2 de *C. gattii* como

transportadores de alta e baixa afinidade por zinco, respectivamente, e avaliou seu impacto na virulência desse patógeno (SCHNEIDER *et al.*, 2015). *C. gattii* ainda possui outros dois transportadores da família ZIP, sendo Zip3 um ortólogo ao Atx2 de *S. cerevisiae*.

De acordo com o YeastDB (JM *et al.*, 2012) há, no presente momento, 264 genes cujos mutantes (nocaute, superexpressão, e outros) estão anotados com termos associados a homeostase de manganês: tolerância alterada a metais, resistência alterada a químicos, transporte de pequenas moléculas alterado, assim como menores taxas de crescimento. Como esses genes são conservados entre células fúngicas, a observação dessas alterações fenotípicas abre um novo caminho para o desenvolvimento de estratégias de controle a doenças fúngicas, bem como maior entendimento sobre interações na interface patógeno-hospedeiro.

2. OBJETIVOS

2.1 Objetivo geral

Analisar a importância de sistemas reguladores da homeostase de manganês na levedura patogênica *Cryptococcus gattii* e sua relação com a virulência.

2.2 Objetivos específicos

2.2.1 Capítulo 1

- Construção de linhagem mutante nula e complementada para o gene *ZIP3* (CNBG_5361) de *C. gattii*
- Observar os efeitos da ausência do transportador Zip3 na tolerância a manganês e sensibilidade a agentes formadores de espécies reativas de oxigênio em *C. gattii*
- Mensurar o acúmulo de espécies reativas de oxigênio nas linhagens mutantes nula e complementada, bem como na linhagem selvagem
- Avaliar a expressão de genes cujos produtos estão envolvidos no transporte de metais em compartimentos intracelulares e na resposta a estresse oxidativo na linhagem mutante nula *zip3* comparada a linhagem selvagem
- Quantificar manganês intracelular nas linhagens mutantes nula e complementada bem como na linhagem selvagem
- Avaliar o desfecho da interação entre as linhagens mutantes nula e complementada, bem como a linhagem selvagem com uma linhagem de macrófagos
- Avaliar o papel do transportador Zip3 na virulência de *C. gattii* em modelo não-mamífero de infecção

- Avaliar o papel do transportador Zip3 na secreção de determinantes de virulência clássicos de *C. gattii*, como glicuronoxilomanana, vesículas extracelulares e melanina

2.2.2 Capítulo 2

- Construção de linhagens mutantes nulas para o gene *SMF1* (CNBG_6162) de *C. gattii*
- Avaliar o impacto da ausência do transportador Smf1 em *C. gattii* na aquisição de metais
- Avaliar a expressão do gene *SMF1* de *C. gattii* frente a diversas concentrações de manganês
- Observar os efeitos da ausência do transportador Smf1 na sensibilidade a agentes formadores de espécies reativas de oxigênio em *C. gattii*
- Avaliar o papel do transportador Smf1 na virulência de *C. gattii* em modelo não-mamífero de infecção

3. RESULTADOS

Os resultados obtidos estão apresentados na forma de dois manuscritos. O primeiro versa sobre o papel do transportador Zip3 na aptidão celular e virulência de *C. gattii*. O segundo capítulo descreve a influência do transportador Smf1 na aquisição de metais e virulência de *C. gattii*. O capítulo um se encontra publicado no periódico Fungal Genetics and Biology. O segundo está redigido na forma de artigo regular e formatado conforme regras do periódico Fungal Genetics and Biology a ser submetido. As figuras foram dispostas ao longo do texto com suas respectivas legendas a fim de facilitar a leitura e interpretação dos resultados.

3.1 Capítulo 1

Participation of Zip3, a ZIP domain-containing protein, in stress response and virulence in *Cryptococcus gattii*

3.2 Capítulo 2

The role of the Nramp transporter of *Cryptococcus gattii* in metal acquisition and virulence

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Participation of Zip3, a ZIP domain-containing protein, in stress response and virulence in *Cryptococcus gattii*

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ABSTRACT

Cryptococcus gattii is an etiologic agent of cryptococcosis, a potentially fatal disease that affects humans and animals. The successful infection of mammalian hosts by cryptococcal cells relies on their ability to infect and survive in macrophages. Such phagocytic cells present a hostile environment to intracellular pathogens via the production of reactive nitrogen and oxygen species, as well as low pH and reduced nutrient bioavailability. To overcome the low-metal environment found during infection, fungal pathogens express high-affinity transporters, including members of the ZIP family. Previously, we determined that functional zinc uptake driven by Zip1 and Zip2 is necessary for full *C. gattii* virulence. Here, we characterized the *ZIP3* gene of *C. gattii*, an ortholog of the *Saccharomyces cerevisiae ATX2*, which codes a manganese transporter localized to the membrane of the Golgi apparatus. Cryptococcal cells lacking Zip3 were tolerant to toxic concentrations of manganese and had imbalanced expression of intracellular metal transporters, such as the vacuolar Pmc1 and Vcx1, as well as the Golgi Pmr1. Moreover, null mutants of the *ZIP3* gene displayed higher sensitivity to reactive oxygen species (ROS) and substantial alteration in the expression of ROS-detoxifying enzyme-coding genes. In line with these phenotypes, cryptococcal cells displayed decreased virulence in a non-vertebrate model of cryptococcosis. Furthermore, we found that the *ZIP3* null mutant strain displayed decreased melanization and secretion of the major capsular component glucuronoxylomannan, as well as an altered extracellular vesicle dimensions profile. Collectively, our data suggest that Zip3 activity impacts the physiology, and consequently, several virulence traits of *C. gattii*.

1. Introduction

Cryptococcus gattii is an etiologic agent of cryptococcosis, a potentially fatal disease that affects humans and animals. The disease is characterized by a primary lung infection that may disseminate to the whole organism, leading ultimately to meningoencephalitis (Kronstad et al., 2011). There are an estimated 220,000 cryptococcal meningitis cases a year, with > 80% mortality associated based on the geographic localization (Rajasingham et al., 2017). Despite the large majority of cryptococcosis cases being derived from *C. neoformans* infections, *C. gattii* is gaining more attention due to its ability to infect immunocompetent hosts (Akins and Jian, 2019). Isolates from *C. gattii* are endemic in many countries, and are commonly associated with eucalyptus trees in tropical and subtropical regions (Correa Pinheiro et al., 2019; Herkert et al., 2017).

Successful infection by cryptococcal cells relies on their ability to

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survive in distinct phagocytes by producing several virulence determinants that modulate the activity of these host cells (Leopold Wager et al., 2016). Such phagocytic cells can present a hostile environment to intracellular pathogens via the production of reactive nitrogen and oxygen species, and decreased nutrient bioavailability (Gilbert et al., 2015; Leopold Wager et al., 2016). The strategy of kidnapping nutrients away from the pathogen is essential for eliminating many microorganisms, a process known as nutritional immunity (Hood and Skaar, 2012). Transition metals such as zinc, iron, and manganese are pivotal to many biological processes, making them interesting targets for sequestration (Malavia et al., 2017).

The intracellular metal levels are tightly regulated by the expression of several ion transporters. These proteins are associated in organelles or the cell membrane and provide ion exchange according to the needs of the cell and to avoid the potential effects of metals overload (Bowers and Srai, 2018; Guo et al., 2016; Takagishi et al., 2017). During infection, pathogens such as fungi and bacteria encounter a low-metal environment and express high-affinity transporters to overcome the starvation of such ions to survive and proliferate (Begg, 2019; Gerwien et al., 2018; Malavia et al., 2017).

Manganese is important for a variety of biochemical processes, from central metabolism to oxidative stress in eukaryotic and prokaryotic cells, contributing to cell viability and virulence in the host (Kelliher and Kehl-Fie, 2016). This transitional metal is a cofactor for enzymes involved in deoxynucleotide biosynthesis, oxidases, dehydrogenases (MnSOD), kinases, decarboxylases, and sugar transferases (Culotta et al., 2006, 2005; Reddi et al., 2009). Moreover, several fungal pathogens as *C. neoformans*, *C. gattii*, *Malassezia sympodialis*, *Aspergillus fumigatus*, and *Candida albicans* express MnSOD as a strategy against the oxidative burst of phagocytes (Fréalle et al., 2005). In *S. cerevisae*, Smf1 is the main transporter in extracellular manganese uptake, localized in plasma membrane and endosomes. Furthermore, Smf2, Ccc1 and Pmr1 also contribute to manganese homeostasis maintenance, promoting manganese import to organelles as endoplasmic reticulum, vacuole and Golgi apparatus, respectively (Culotta et al., 2005). In contrast to Pmr1 function, Atx2 is a manganese exporter in the Golgi apparatus, increasing manganese intracellular levels (Lin and Culotta, 1996).

The ZIP family of transporters is conserved among the domains of life and has been characterized in some models. These proteins transport zinc, iron, and manganese to the cytoplasm from either the extracellular space or from inside organelles (Jeong and Eide, 2013). Our group has previously demonstrated the importance of ZIP transporters in fungal virulence for *C. gattii*. The proteins Zip1 and Zip2 were expressed in response to zinc deprivation, and *zip1* null mutants displayed reduced growth in zinc-deprived media and inside phagocytes. Moreover, the absence of both Zip1 and Zip2 led to impaired virulence in murine model *of* cryptococcosis (Ribeiro et al., 2017; Schneider et al., 2015).

To systematically characterize the set of genes coding for ZIP transporters in *C. gattii*, we evaluated the *ZIP3* gene. This gene is an ortholog to *Saccharomyces cerevisiae ATX2*, whose product is characterized as a manganese transporter located in the Golgi membrane. Null mutants of *ATX2* displayed a decrease in intracellular manganese levels, while overexpression mutants had increased manganese levels. Moreover, yeast cells lacking Atx2p displayed increased capability to grown in toxic concentrations of manganese compared to WT cells. As manganese ions are known to be associated with cellular redox metabolism, epistasis analysis revealed that *ATX2* can suppress SOD1 deficiency in yeast cells (Lin and Culotta, 1996). While the homeostasis of iron, zinc, and copper is relatively well elucidated in cryptococcal cells, little is known about the set of proteins that regulates manganese metabolism. Here, we present a functional characterization of the *ZIP3* gene of *C. gattii* and its impact on several virulence traits.

2. Materials and methods

2.1. Bioinformatic analyses

Protein sequences were retrieved from FungiDB database (Basenko et al., 2018) using as query the presence of the ZIP conserved domain (PFAM code PF02535). Prediction of transmembrane helices were performed using the TMHMM (Krogh et al., 2001) platform in FungiDB. Fungal ZIP proteins were aligned using MAFFT (Katoh et al., 2019) using default parameters. The evaluation of best evolutive models for Maximum likelihood (ML) test of phylogeny was conducted with MEGA X (Kumar et al., 2018) and the phylogenetic analysis were finally conducted with PhyML (Guindon et al., 2009), using 500 bootstraps to measure the branch support.

2.2. Strains and culture conditions

Cryptococcus gattii R265 and *Escherichia coli* TG2 were used in this work. Fungal strains were routinely cultured in YPD medium (2% glucose, 2% peptone, and 1% yeast extract) incubated at 30 °C on a constant rotation platform at 200 rpm. Agar was added at a final concentration of 1.5% when solid media was used. YNB (yeast nitrogen base) without amino acids, supplemented with 2% glucose and asparagine (40 mM), was used for the phenotypic assays.

2.3. Null and complemented strain construction

The DelsGate methodology (García-Pedrajas et al., 2008) was used to construct the *ZIP3* mutant strains. For null strain cassette construction (*zip3*), the 5′ and 3′ flanking regions (~1000 bp) of the ZIP3 gene (CNBG_5361) were PCR-amplified and gel-purified using a Illustra GFX PCR DNA and Gel Band Purification Kit (GE Healthcare). Both fragments were mixed (~30 ng each) with pDONR-NAT vector (~300 ng) (Schneider et al., 2012) and submitted to BP Clonase reaction according to the manufacturer's instructions (Invitrogen). For the complemented strain (*zip3::ZIP3)*, the *ZIP3* coding region was PCR-amplified and cloned into the *Eco*RV site of pJAF15 vector, which carries the hygromycin-selectable marker. Both cassettes were transformed in E. coli TG2 cells and selected by antibiotic resistance screening and colony PCR. Biolistic transformation was performed to introduce the deletion construct previously linearized by I-SceI enzymatic digestion in *C. gattii*. For the complemented strain, the biolistic transformation was performed into the *zip3* mutant strain using *Not*I-digested complementation plasmid and expected to be randomly integrated into the genome. The mutant strains were confirmed by Southern blot analysis and semi-quantitative reverse transcription (RT)-PCR. Supplementary Table 1 lists the primers used for the plasmid construction and screening.

2.4. RT-qPCR analysis

For RNA extraction, the *C. gattii* R265 and *zip3* null mutant strain were pre-inoculated in YPD medium overnight. The cells were then washed three times with phosphate-buffered saline (PBS 1X) and counted in a Neubauer chamber. A total of 1×10^7 cells were inoculated in YNB medium (control) or YNB containing $MnSO_4$ (1 mM). The cells were incubated for 2 h at 30 °C. RNA was isolated using TRIzol (Invitrogen) after cell lysis with liquid nitrogen using a mortar and pestle. RNA integrity and concentration were evaluated by electrophoresis on 1% agarose gel and by fluorometry using a Qubit fluorometer and Quant-iT RNA Assay Kit according to the manufacturer's instructions (Invitrogen). Complementary DNAs (cDNAs) were prepared using DNase (Promega)-treated total RNA samples (1.000 ng) with ImProm-II Reverse Transcriptase (Promega) using oligo-dT. Quantitative RT-PCR (qRT-PCR) was performed on a StepOne Real-Time PCR System (Applied Biosystems) with thermal cycling conditions set with an initial step at 95 °C for 10 min, followed by 40 cycles at

95 °C for 15 s, 60 °C for 15 s, and 72 °C for 60 s. The PCR were conducted at a final volume of 20 μL, containing 2 μL cDNA (2.5 ng/μL), 2 μL SYBR Green (1:1000, Invitrogen), 0.1 μL dNTP (5 mM), 2 μL PCR buffer 10×, 1.2 μL $MgCl_2$, 0.05 U Platinum Taq DNA Polymerase (Invitrogen), and 0.2 μL each primer (5 pmol). Melting curve analysis was performed at the end of the reaction to confirm the presence of a single PCR product. All experiments were performed using three independent cultures, and each cDNA sample was analyzed in triplicate for each primer pair. Data were normalized to actin cDNA amplified in each set of PCR experiments. Relative expression was determined by the comparative threshold cycle ($2^{-\Delta\Delta\ CT}$) method (Schmittgen and Livak, 2008). Supplementary Table 1 lists the primers used for quantifying each cDNA level.

2.5. Spot plate assay

The wild-type (WT), *zip3* null mutant, and *zip3::ZIP3* complemented strains were cultivated in YPD medium overnight at 30 °C under agitation (200 rpm) and washed three times with PBS. A suspension of cells (optical density at 600 nm wavelength, OD_{600} = 1.0) from each strain was prepared and serially diluted up to 10,000 × . A 3 μL aliquot of each suspension was spotted on YNB medium or YNB medium containing 100 μM DTPA (diethylenetriamine pentaacetic acid). The plates were incubated at 30 °C for 2 days and photographed.

2.6. Growth assays

After overnight culture of WT, *zip3* null mutant, and *zip3::ZIP3* complemented strains in YPD medium at 30 °C, the cells were collected by centrifugation and washed three times with PBS. The cell density was determined using a Neubauer chamber. A total of 20,000 cells were suspended in 100 μL YNB and distributed into 96-well plates. An additional 100 μL YNB (control) or YNB supplemented with increasing concentrations of $MnSO_4$ (final concentration 10 mM), menadione (final concentration 5 μg/mL), paraquat (final concentration 1 mM), diethyl maleate (final concentration 0.3 mM), or the calcineurin inhibitor FK-506 (final concentration, 1 μg/mL) were also added. Higher concentrations of diethyl maleate could not be evaluated due to solubility issues. The plates were incubated for 24 h at 30 °C or 37 °C, and the OD_{600} was determined using a SpectraMax i3 microplate reader.

2.7. Manganese determination

The *C. gattii* WT, *zip3* and *zip3::ZIP3* strains were inoculated into YPD medium and allowed to grow at 30 °C for 18 h. After washing with PBS, approximately 1 ×10^9 cells of each strain were inoculated in YNB or YNB added of 1 mM $MnCl_2$ and incubated for further 2 h at 30 °C on a constant rotation platform (200 rpm). Cells were centrifuged and washed with ice cold ultrapure water containing 1 mM EDTA. Each cell pellet was then treated using a conventional wet digestion method for sample preparation by the addition of 400 μL of bidistilled concentrated HNO_3 and 100 μL of 30% H_2O_2. The samples were then heated (95 ± 5 °C) for 1 h and the final volume was set to 5 mL using ultrapure water. A blank digested sample (without sample) was also carried through the procedure.

A PerkinElmer Model Pin AACle 900 T atomic absorption spectrometer equipped with an AS 900 autosampler and a transversely heated graphite tube furnace (THGA) and Zeeman-effect background correction with a transverse magnetic field was used. Hollow cathode lamp of Mn was used as a radiation source operated under a current of 20 mA. The signal was evaluated by the integrated absorbance (peak area) in the analytical line of 279.48 nm, with a spectral slit of 2.0 nm. The instrumental conditions were those indicated by the manufacturer.

2.8. Melanization assay

The yeast strains were pre-cultivated in YPD medium overnight at 30 °C and washed three times with PBS. A total of 4 × 10^6 cells were then inoculated in 10 mL melanization medium (0.5 g/L $MgSO_4{\cdot}7H_2O$, 3 g/L KH_2PO_4, 1 mg/L thiamine, 1 g/L asparagine, and 1 mM L-DOPA) and incubated for 96 h at 37 °C with 200 rpm agitation, protected from light. Every 24 h, a 1 mL aliquot was removed, and the cells were collected by centrifugation. The OD_{400} was determined from the supernatant using the SpectraMax i3 (Molecular Devices) microplate reader. For the spot plate assay, a suspension of cells (OD_{600} = 1.0) from each strain was prepared, and a 3 μL aliquot from each suspension was spotted on melanization medium containing 1.5% agar and incubated at 37 °C for 6 days.

2.9. Microscopy analysis

Capsule formation was examined using light microscopy to visualize India ink counterstaining of cells collected after 24-h incubation of the yeast strains (37 °C and 5% CO_2) in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum (FBS) that had been prepared with India ink. Relative capsule sizes were defined as the ratio between the capsule thickness and cell diameter. The capsule measurements of 100 cells from each strain were determined using ImageJ. The distribution of chitin-like structures in the cell surface and the cell wall was evaluated using confocal laser scanning microscopy (Olympus FluoView 1000 confocal laser scanning microscope at the Universidade Federal do Rio Grande do Sul Electron Microscopy Center [CME – UFRGS]). This was performed by staining the cells with Alexa Fluor 594 conjugate of wheat germ agglutinin (WGA) and calcofluor white as previously described (Ribeiro et al., 2017).

2.10. Evaluation of secreted glucuronoxylomannan and extracellular vesicles

The WT, *zip3* null mutant, and *zip3::ZIP3* complemented strains were cultured in DMEM, as performed for capsule visualization. After 48 h, a 1-mL aliquot was removed, and the cells collected by centrifugation. The glucuronoxylomannan (GXM) concentration in the supernatant was assayed using mouse anti-GXM immunoglobulin (Ig)G1 18B7 as previously described (Casadevall et al., 1992). The concentration and size distribution of extracellular vesicles (EVs) were evaluated after these membrane structures had been isolated following *C. gattii* growth on solid YPD medium for 24 h. After sequential centrifugation to remove cells and debris, and ultracentrifugation for collecting the EVs, the samples were analyzed by nanoparticle tracking analysis (NTA) using an LM10 nanoparticle analysis (Malvern) system coupled with a 488-nm laser as recently described (Reis et al., 2019).

2.11. Reactive oxygen species measurements

The cells were cultured overnight in YPD medium at 30 °C on a constant rotation platform (200 rpm). The cells were washed three times using PBS, and an inoculum of 1 × 10^7 cells/mL was suspended in YNB medium. After 2 h incubation at 30 °C on a constant rotation platform, 1 mL culture was collected, washed with PBS, and CM-H_2DCFDA (Invitrogen) was added to a final concentration of 10 μM. The cells were incubated in the dark for 1 h, washed with PBS, and analyzed for fluorescence determination utilizing the SpectraMax i3 with the emission and excitation wavelenghts set at 488 and 520 nm, respectively. Fluorescence values were normalized to cell count, based on OD_{600} determination.

2.12. Macrophage assays

The susceptibility of the mutant strains to macrophage activity and

the resistance of the strains to the antifungal action of phagocytes were evaluated. One day before the co-incubation, an aliquot of 1×10^5 J774.16 cells (from three to ten passages) in DMEM supplemented with 10% FBS was seeded into 96-well culture plates and incubated at 37 °C with 5% CO_2.

The *C. gattii* strains were inoculated into YPD medium and allowed to grow at 30 °C for 18 h. The next day, the fungal cells were washed three times with PBS and opsonized with anti-GXM antibody 18B7 (final concentration, 1 μg/mL), and incubated for 1 h at 37 °C. Macrophages were activated with phorbol myristate acetate (PMA) at a final concentration of 50 nM in FBS-free DMEM for 1 h at 37 °C with 5% CO_2. After opsonization, a total of 1×10^6 cells/mL were added to the wells containing the macrophages and incubated for 2 h at 37 °C with 5% CO_2. After the incubation period, the wells were washed three times with PBS, and one set of wells was treated with 0.1% Triton X-100 to lyse macrophage-like cells, and the fungal loads were determined by colony forming unit (CFU) analysis in YPD medium. The other set of washed wells received 100 μL of fresh DMEM and was further incubated at 37 °C with 5% CO_2. After 24-h incubation, the wells were washed three times with PBS and treated with 0.1% Triton X-100 to lyse macrophage-like cells. The fungal loads in the macrophages were determined and the intracellular proliferation rate (IPR) was determined as the ratio between the fungal loads at 24 and 2 h.

2.13. Virulence assays in Galleria mellonella

Galleria mellonella larvae (15–20 mm and 220–260 mg, n = 10) with no signs of infection, such as the absence of grey pigmentation, were infected by injection into the last proleg with 10 μL 10^5 cells from the WT, *zip3* null mutant, and *zip3::ZIP3* complemented strain suspensions previously cultured in YPD medium on a shaking platform (30 °C). The same volume of sterile PBS or dimethyl sulfoxide (DMSO) was used as controls. All larvae were placed in sterile Petri dishes and maintained at 37 °C with daily monitoring of mortality for up to 10 days.

2.14. Statistical analysis

Statistical differences were analyzed using an unpaired *t*-test or one-way analysis of variance (ANOVA). All values are reported as means and the standard error (SD). Survival curves were analyzed using the log-rank (Mantel–Cox) test.

3. Results

3.1. In silico characterization of C. gattiig Zip3

In order to gain insights about functions associated with the *ZIP3* product, we performed a set of *in silico* predictions. According to FungiDB (Basenko et al., 2018), Zip3 is an ortholog of the *S. cerevisiae* Atx2p (Ortholog group OG5_130597). This protein is associated with manganese metabolism and transports such ions from the Golgi to cytoplasm (Lin and Culotta, 1996). The similarity between Zip3 and Atx2p sequences is about 42% in almost 80% of the protein length. Zip3 displays 7 transmembrane domains and the presence of the ZIP conserved domain (PFAM accession PF02535) (Fig. 1A), as revealed by TMHMM and InterProScan analyses in the FungiDB. Moreover, both Atx2p and Zip3 were predicted to be localized in the secretory pathway, according to the YLoc prediction server (Briesemeister et al., 2010), and in the endomembrane system, according to the BUSCA prediction server (Savojardo et al., 2018). A phylogenetic analysis was performed using ZIP domain-containing proteins from *S. cerevisiae, C. albicans* and *C. gattii*, as some members of this set of proteins have already been functionally characterized. A clear separation in three distinct clusters could be observed, being Zip3 grouped with Atx2 orthologs. Other cryptococcal ZIP domain-containing proteins cluster with their respective orthologs/paralogs (Fig. 1B). Collectively, these predictions suggest that cryptococcal Zip3 may develop similar functions to those associated with *S. cerevisiae* Atx2p.

3.2. Evaluation of Zip3 participation in cryptococcal metal homeostasis

We have previously demonstrated that extracellular metal chelation by DTPA (100 μM) led to a slight increase in *ZIP3* transcript levels (Schneider et al., 2015). To evaluate whether Zip3 may be involved in zinc uptake, we first generated null (*zip3*) and complemented mutants (*zip3*::*ZIP3*) of the *ZIP3* gene. The presence of inactivation and complementation alleles were confirmed by PCR and RT-PCR (data not shown). In addition, we confirm that the complementation cassette was randomly integrated into the *zip3* null mutant as the complement strain retains resistance to both nourseothricin (from the inactivation cassette) and hygromycin (from the complementation cassette). We then evaluated their sensitivity to DTPA. We could not find differences in the growth capabilities of the *zip3* null mutants compared to the WT and *zip3*::*ZIP3* strains in the presence of this metal chelator (Fig. 2A). We next sought to determine if *C. gattii* Zip3 shares functions with *S. cerevisiae* Atx2p. Mutants lacking *ATX2* display increased tolerance of $MnCl_2$ (Lin and Culotta, 1996). As expected, the same phenotypic alteration could be found for the *C. gattii zip3* null strains over a range of $MnSO_4$ concentrations, but not in the WT and *zip3::ZIP3* complemented strains (Fig. 2B). To understand the molecular mechanisms associated with this tolerance of high concentrations of manganese, we evaluated the expression of *VCX1*, *PMC1*, and *PMR1*, whose products possibly mediate mobilization of excessive manganese to cellular compartments, as determined for their orthologs in *C. neoformans* and *S. cerevisiae* (Antebi and Fink, 1992; Kmetzsch et al., 2013, 2010), as well as for *ZIP3*. Such experiments were performed using an exposure for 2 h to 1 mM $MnSO_4$ in order to evaluate the early set of genes expressed to a condition that displayed mild toxicity to WT cells. The expression of *ZIP3, VCX1*, *PMC1*, and *PMR1* did not change in WT cells under such conditions compared to control. However, when the null mutants were incubated with manganese in the same conditions, we observed increased expression of the Golgi-associated manganese importer coded by *PMR1* (Fig. 2C). It is worth noting that, irrespective of the presence of manganese, cells lacking *ZIP3* displayed decreased levels of *PMC1* and *VCX1* compared to WT cells.

We then evaluated the total manganese content in WT, *zip3*, and *zip3::ZIP3* strains cultured in YNB or YNB containing 1 mM $MnCl_2$. The addition of manganese to the culture medium led to an approximate 10-fold change in the cellular associated manganese content. However, *zip3* null strains displayed decreased levels compared to the WT strain, irrespective of the presence of manganese in the culture medium (Table 1). Collectively, these data suggest that Zip3 could participate in the homeostasis of manganese in cryptococcal cells.

3.3. Zip3 participates in the cryptococcal stress response

As demonstrated for *S. cerevisiae,* manganese mobilization from the Golgi is important for developing an antioxidative response (Lin and Culotta, 1996). We then evaluated whether the absence of *ZIP3* in *C. gattii* would cause an imbalance in redox homeostasis. First, we evaluated the abundance of reactive oxygen species (ROS) in WT, *zip3* null strain and *zip3::ZIP3* complemented strain using the intracellular fluorescent ROS probe CM-H_2DCFDA. We could identify higher fluorescent signal content in the *zip3* null mutants compared to the WT and complemented strains (Fig. 3A), confirming our hypothesis that Zip3 participates in cryptococcal redox homeostasis. To understand the mechanisms associated with the accumulation of ROS due to the absence of *ZIP3*, the expression of two catalase (CAT)-coding genes (*CAT1*, CNBG_4696; *CAT2*, CNBG_5786), two superoxide dismutase (SOD)-coding genes (*SOD1*, CNBG_0599; *SOD2*, CNBG_2661), and two glutathione peroxidase (GPX)-coding genes (*GPX1*, CNBG_4202; *GPX2*, CNBG_5153) were evaluated using RNA collected from WT and *zip3*

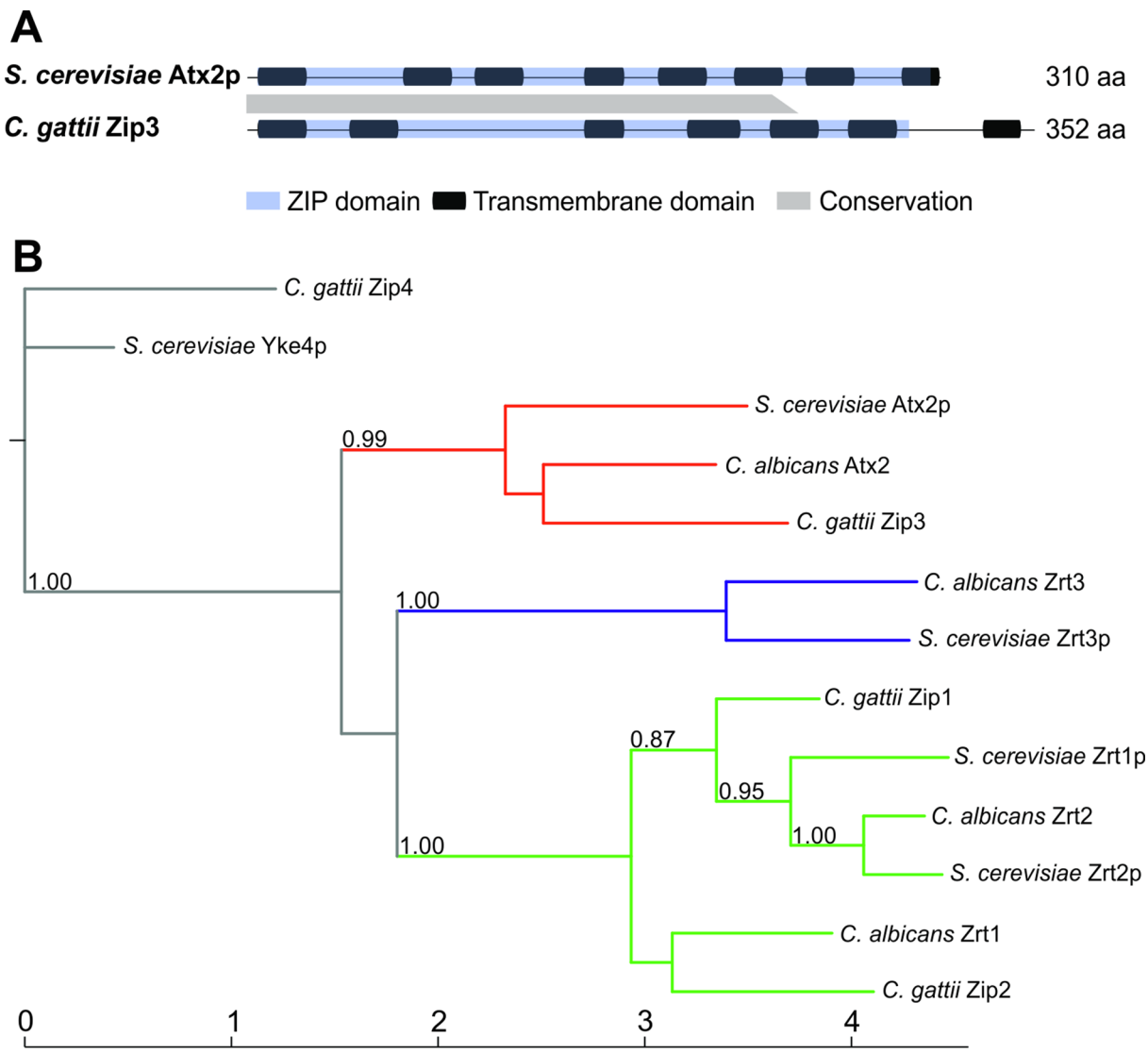

Fig. 1. In silico characterization of *C. gattii* Zip3. (A) Comparison between *C. gattii* Zip3 and *S. cerevisiae* Atx2p demonstrating the common presence of ZIP domain (blue), transmembrane domains (black boxes), as well as a large segment of high identity of similarity (grey box). The length of each protein sequence (in amino acids) is indicated to the right. (**B**) Phylogenetic analysis applying the ML method and including ZIPs sequences from *C. gattii* (Zip1 - CNBG_6066, Zip2 - CNBG_2209, Zip3 - CNBG_5361, and Zip4 - CNBG_3633), *C. albicans* (Zrt1 - C4_06970C_A, Zrt2 - C2_02590W_A-T, Zrt3 - C2_02180W_A, and Atx2 - C3_00390W_A), and *S. cerevisiae* (Zrt1p, Zrt2p, Atx2p, Yke4p). Only branches with high confidence of bootstrap (over 80%) are annotated. The bar marker indicates the genetic distance, which is proportional to the number of amino acid substitutions.

null mutant cells. The absence of *ZIP3* led to decreased expression of the *SOD* genes (fold change of 0.02 and 0.01 for *SOD1* and *SOD2* in the *zip3* strain compared to WT, respectively). However, the opposite pattern was found for the *CAT* (fold change of 2.36 and 9.69 for *CAT1* and *CAT2* in the *zip3* strain compared to WT, respectively) and *GPX* genes (fold change of 31.25 and 45.86 for *GPX1* and *GPX2*) in the *zip3* strain compared to WT, respectively). We also evaluated the impact of manganese on the expression of such genes in both strains. A decreased expression could be observed in the *SOD1* levels when comparing the WT in control and manganese conditions (fold change of 1.68 times). A similar pattern of expression could be observed for the *CAT2* gene in the *zip3* null mutants background (fold change of 1.57 times) (Fig. 3B). Collectively, these results allowed us to assume that lack of *ZIP3* led to dysregulated expression of genes required for defenses to oxidative stress.

As imbalanced ROS homeostasis was found in cells lacking *ZIP3*, we evaluated the sensitivity of the WT, *zip3* null mutants, and *zip3*::*ZIP3* complemented strains to reactive species generators. The null mutants displayed increased sensitivity to paraquat (Fig. 4A), menadione (Fig. 4B), as well as to the glutathione-depleting agent diethyl maleate (Fig. 4C), which ultimately drives the accumulation of ROS. In *S. cerevisiae*, *sod1 atx2* double null mutant cells are dependent of exogenously added manganese in order to adapt to ROS stress (Lin and Culotta, 1996).We then hypothesize that a similar phenotype could be observed in the *C. gattii zip3* null mutant. We evaluate the growth of WT, *zip3* null mutant, and *zip3*::*ZIP3* complemented strains in YNB supplemented with $MnSO_4$ (0.5 mM and 1 mM) added or not of Paraquat (0.25 mM). A clear recovery of growth capabilities could be observed of *zip3* null mutants when manganese was added to YNB containing paraquat (Fig. 4D).

We also evaluated whether the absence of *ZIP3* would influence the Ca^{2+}–calcineurin pathway, an important regulator of the expression of metal channels, such as Pmc1, Pmr1, Vcx1, as well as the responses to distinct stressors (Chow et al., 2017; Kmetzsch et al., 2013, 2010). The sensitivity of the WT, *zip3* null mutant, and *zip3*::*ZIP3* complemented strains to a pharmacological calcineurin inhibitor (FK-506) was evaluated by analyzing the growth rates at distinct concentrations of FK-506. The absence of *ZIP3* led to decreased growth capabilities in 0.04

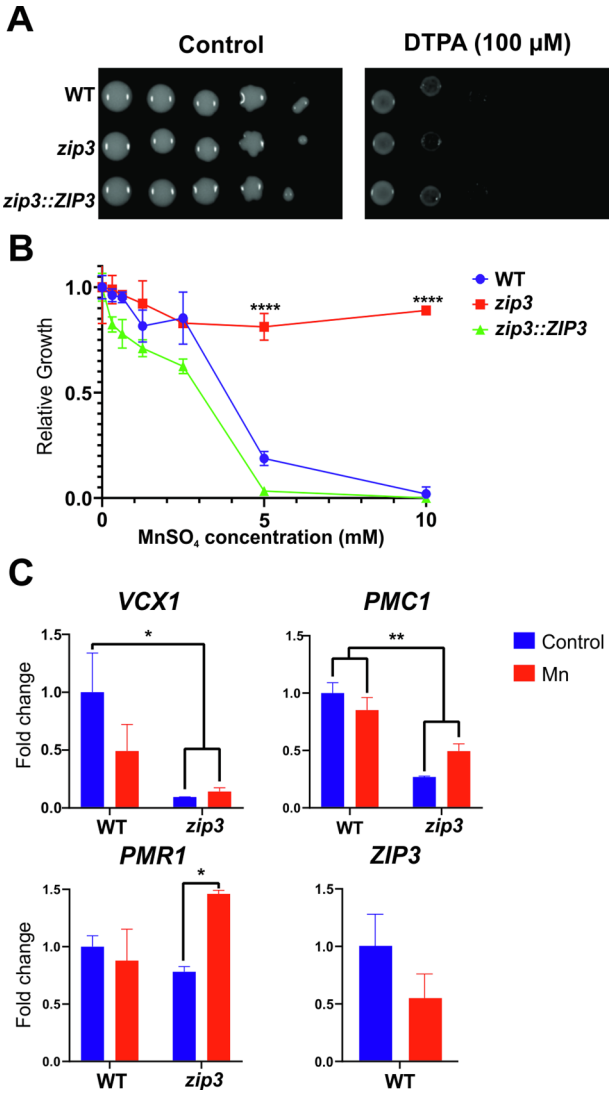

Fig. 2. Zip3 displayed altered metal homeostasis. (A) A suspension of cells (OD_{600} = 1.0) from each strain was prepared and serially diluted up to 10,000 times. A 3 μL aliquot of each suspension was spotted on YNB medium or YNB medium containing 100 μM of the metal chelator DTPA. The plates were incubated at 30 °C for 2 days and photographed. (**B**) Measurement of WT, *zip3*, and *zip3::ZIP3* growth after 24 h in YNB medium (control) or YNB supplemented with increasing concentrations of $MnSO_4$ by OD_{600} determination. **(C)** RT-qPCR analysis of the vacuolar transporter–coding genes *VCX1* and *PMC1*, the Golgi importer–coding gene *PMR1*, and the *ZIP3* gene. A total of 10^7 cells from each strain were inoculated in YNB medium (control) or in YNB containing 1 mM $MnSO_4$ for 2 h at 30 °C. The measured quantity of the mRNA in each sample was normalized using the Ct values obtained for the *ACT1* gene. Data are the means and SD of three biological replicates. Two-way ANOVA followed by Tukey's multi-comparison test was performed. The asterisks denote statistically significant differences (*P < 0.05; **P < 0.01).

and 0.06 μg/mL FK-506 compared to the WT and *zip3*::*ZIP3* complemented strains. However, higher concentrations (0.1–1 μg/mL) of the calcineurin inhibitor caused a similar pattern of toxicity to all strains (Fig. 4E), suggesting partial participation of Zip3 in Ca^{2+}–calcineurin pathway regulation.

3.4. Cryptococcal virulence is affected by the absence of ZIP3

As null mutants of the *ZIP3* gene displayed imbalanced ROS metabolism and hypersensitivity to the inhibition of a stress response pathway, we hypothesized that Zip3 would also affect cryptococcal virulence. We first evaluated whether Zip3 would alter the outcome from the interaction with macrophages. We found that the *zip3* null mutants displayed decreased fungal loads after 2 h of co-incubation with J774.16 macrophage-like cells compared to the WT and the *zip3*::*ZIP3* complemented strain (Fig. 5A). To evaluate whether the cryptococcal cell surface structures that mediate recognition by phagocytic cells would be altered by the absence of *ZIP3*, we evaluated the capsule size and distribution of chitooligomers. No differences in such characteristics could be found when comparing the WT and *zip3* null mutants (Supplementary Fig. 1).

We next determined the capability of surviving inside macrophages and found that this phenotype did not depend on Zip3 activity (Fig. 5B). Therefore, we sought to evaluate the effect of the absence of *ZIP3* in a non-mammalian model of cryptococcosis. Infection of *G. mellonella* with the WT and *zip3*::*ZIP3* complemented strains caused death in 50% of the larvae (LT_{50}) in 8.5 and 10 days, respectively (Fig. 5C). However, only 30% of the larvae infected with the *zip3* null mutants died in the period analyzed (10 days), confirming a consistent decrease in virulence compared to the WT (P = 0.0002) and *zip3*::*ZIP3* complemented strain (P = 0.0213).

To identify the mechanisms associated with the decreased virulence of *C. gattii* in the absence of Zip3, we evaluated the main cryptococcal virulence determinants. We used enzyme-linked immunosorbent assay (ELISA) to determine the concentration of the major capsular component GXM in the supernatants of WT, *zip3* null mutants, and *zip3*::*ZIP3* complemented strains cultured in DMEM for 48 h. A higher concentration of GXM was found in the cells lacking *ZIP3* compared to the WT and complemented strains (Fig. 6A).

We also evaluated extracellular vesicles (EVs) abundance and size using a recently developed fast method that is based on their isolation from solid cultures (Reis et al., 2019). We detected a slight increase in the concentration of EVs collected from cells lacking *ZIP3* compared to WT cells (Fig. 6B). However, the complemented strain displayed even higher concentrations of EVs. We speculate that the intricated balance associated with the regulation of EVs release could be affected by the ectopic integration of the complementation cassette in the *zip3* null mutant strain. EVs from both the WT and *zip3*::*ZIP3* complemented strains cultures were distributed in the 100–300-nm size range (average of near 175 nm). However, the population of mutant EVs were distributed in a slight higher size (average of about 200 nm) (Fig. 6C and Supplementary Fig. 2).

The production of the redox active pigment melanin was also evaluated, mainly by detecting a secreted pigment deposited in the cell wall (Camacho et al., 2019). Visual inspection showed decreased melanization of colonies lacking Zip3 (Fig. 6D), and the melanin concentration in the supernatant of these cells was decreased (Fig. 6E). These results link Zip3 activity to the secretion of *C. gattii* virulence determinants.

Table 1
Manganese quantification in *C. gattii* WT, *zip3*, and *zip3::ZIP3* strains.

Strain	Cell manganese content (10^6 atoms per cell) when cultured in	
	YNB	YNB + 1 mM $MnCl_2$
WT	3.5 ± 0.4	36.7 ± 4.8
zip3	2.2 ± 0.8 **	30.0 ± 4.8 *
zip3::ZIP3	3.7 ± 0.4	30.7 ± 4.7

Results are given as mean ± standard deviation. Values with significant differences in the manganese content compared with the WT are marked with asterisks, according to t-Tests: *P < 0.05 and **P < 0.01.

4. Discussion

The ZIP family of transporters is found in all domains of life and plays a crucial role in the uptake of zinc and other metals within the cell

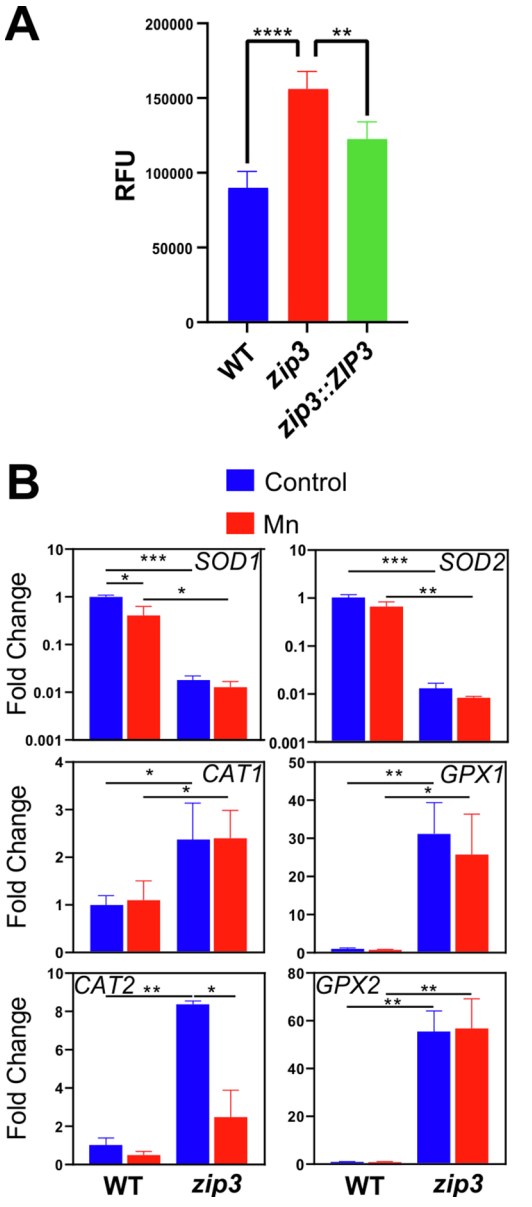

Fig. 3. Impairment of oxidative stress control in cells lacking Zip3. (A) Fluorometric determination of intracellular ROS. A total of 1 $\times 10^7$ cells from each strain were inoculated in YNB medium for 2 h at 30 °C, and then washed and incubated for 1 h with the permeable ROS indicator CM-H_2DCFDA. The cells were then washed and analyzed to quantify the green fluorescence in a SpectraMax i3 system. **(B)** RT-qPCR analysis of two *SOD*-, two *CAT*-, and two *GPX*-coding genes. The measured quantity of the mRNA in each sample was normalized using the Ct values obtained for the *ACT1* gene. Data are shown as the mean and SD of three biological replicates. An unpaired Student *t*-test was performed. The asterisks denote statistically significant differences (*$P < 0.05$; **$P < 0.01$; ***$P < 0.001$).

(Guerinot, 2000). The *ZIP1* and *ZIP2* genes are important in *C. gattii* and *C. neoformans* virulence due to their role in the acquisition of mainly zinc and iron, allowing cryptococcal growth and proliferation even in unfavorable environments (Do et al., 2016; Schneider et al., 2015). However, the Atx2p-like ZIP transporters supposedly act on manganese homeostasis and localize in the membrane of a compartment reminiscent of the Golgi apparatus, as previously demonstrated for *S. cerevisiae* (Lin and Culotta, 1996). Orthologs of Atx2 are well conserved in fungal species, being usually found as a single copy gene in most genomes. An exception are the members of the Schizosaccharomycete class, which has no ATX2 member (Diss et al., 2011; Tamayo et al., 2014).

We previously determined that the *C. gattii ZIP3* gene displayed a slight increase of its transcription levels in response to extracellular metal chelation (Schneider et al., 2015), suggesting that the participation of Zip3 in metal uptake, if in fact exist, should be minor compared to Zip1 and Zip2, whose mutants displayed drastic defects to grow in medium containing zinc chelators. The *zip3* strain exhibited higher tolerance of toxic manganese concentrations, much like its ortholog in *S. cerevisiae* (Lin and Culotta, 1996). Manganese metabolism has been better studied in the budding yeast, in which critical acquisition of the metal comes from intracellular reservoirs and is mediated by proteins such as Atx2p and the NRAMP transporter Smf2p (Culotta et al., 2005). *Cryptococcus neoformans* has only one NRAMP transporter, which is found on the cell membrane and is related to the acquisition of manganese, iron, cobalt, and nickel (Agranoff et al., 2005). As *Cryptococcus* spp. have no ortholog of the main manganese importer Smf2p, cryptococcal Zip3 could play a significant role in raising cytosolic levels of this metal in this pathogen. In line with this assumption, the expression of genes coding the vacuolar transporters Pmc1 and Vcx1, as well of the Golgi importer Pmr1, were altered in the *zip3* mutant. The absence of Zip3 led to decreased expression of the vacuolar transporter–coding genes. This alteration may reflect decreased cytosolic levels of manganese, as Vcx1 orthologs from *S. cerevisiae* are potentially involved in the mobilization of cytosolic excess manganese to vacuoles (Pittman et al., 2004; Pozos et al., 1996). The Golgi transporter Pmr1, which promotes Ca^{2+} and Mn^{2+} transport to the Golgi, may display an antagonistic function to that predicted to Zip3, and was not differentially regulated in the *zip3* null mutant in control conditions, but presented higher levels of transcription in the presence of manganese. These result are in line with the role of Pmr1 in the detoxication of manganese from cells exposed to toxic concentrations of this metal (García-Rodríguez et al., 2015). In this manner, we hypothesize that cells lacking *ZIP3* exposed to excess manganese showed better growth capabilities due to the absence of manganese, supposedly imported from the Golgi. Ultimately, excess manganese is possibly imported by broad range of metal importers and translocated into the Golgi for detoxification by secretion, a process that would be more efficient in the absence of *ZIP3* compared to WT cells. It is noteworthy that manganese itself did not alter the expression of *VCX1*, *PMC1*, and *PMR1* in WT cells. In the sibling species *C. neoformans*, excess of Ca^{2+}, the preferred substrate for these transporters, did not caused increased expression of *VCX1* and *PMR1* (Kmetzsch et al., 2013). This suggests that a balance between the transporters activity could act synergistically to cope with manganese excess toxicity.

In *S. cerevisiae,* Atx2p is associated with redox defense, and its overexpression fully reversed the phenotypes observed in *sod*1Δ cells (Lin and Culotta, 1996). In agreement, the *C. gattii zip3* null mutant had mildly increased intracellular ROS levels compared to the WT. Moreover, we observed a substantial decrease in the expression of genes coding for both SOD isoforms. SODs play a major role in oxidative defense by catalyzing the dismutation of superoxide radical (O_2^-) into oxygen (O_2) or hydrogen peroxide (H_2O_2) (Dias et al., 2006). Sod1 from the sibling species *C. neoformans* is present in the cytoplasm and utilizes zinc and copper as cofactors; its absence is associated with decreased production of virulence determinants, as well as virulence attenuation (Cox et al., 2003; Narasipura et al., 2003). *C. neoformans* Sod2 is found in the mitochondria and uses manganese as a cofactor. Null mutants of *SOD2* were unable to grow at 37 °C, and had higher sensibility to ROS and complete abolition of virulence in a murine model of infection (Narasipura et al., 2005). The increased expression of other oxidative defenses, such as CATs and GPXs, could indicate a compensatory mechanism for addressing increased ROS levels in the *C. gattii zip3* null strains. Accordingly, we also observed higher sensitivity to three different ROS-generating agents in the cells lacking Zip3. Cells lacking *ZIP3* also displayed higher sensitivity to the Ca^{2+}–calcineurin inhibitor FK-506. The Ca^{2+}–calcineurin pathway is activated under stress

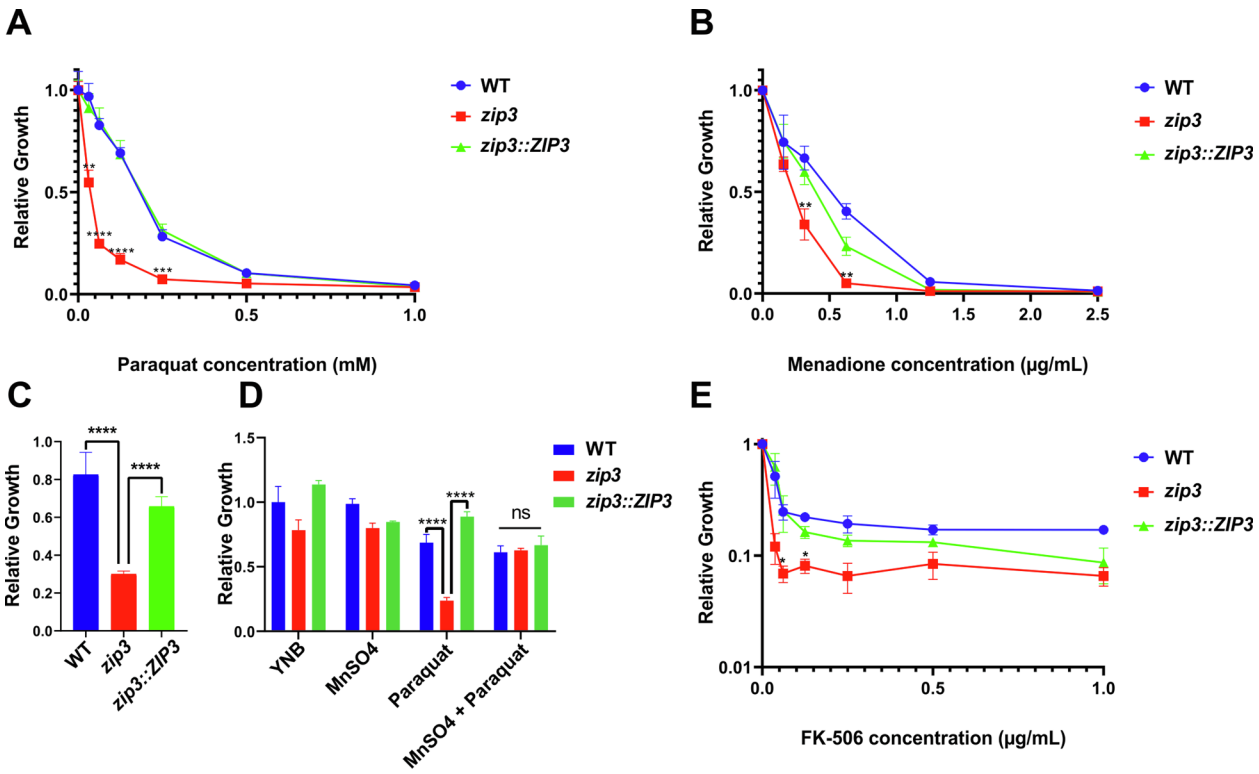

Fig. 4. Zip3 mediates tolerance to oxidative stress. Measurement of WT, *zip3*, and *zip3::ZIP3* growth after 24 h in YNB medium (control) or YNB supplemented with increasing concentrations of the ROS-generating agents **(A)** paraquat and **(B)** menadione, or the **(C)** glutathione-depleting agent diethyl maleate (0.3 mM), or the calcineurin inhibitor **(E)** FK-506. **(D)** Exogenous manganese (0.5 mM) reverts the phenotype of lower growth in *zip3* null strain in presence of paraquat (0.25 μM). Results were normalized by the respective strain in control conditions. Data are the means and SD of three biological replicates. One-way ANOVA followed by Tukey's multi-comparison test was performed. The asterisks denote statistically significant differences compared to the WT strain (*$P < 0.05$; **$P < 0.01$; ***$P < 0.001$; ****$P < 0.0001$).

conditions, and regulates the responses to such conditions by expressing a set of genes, which include those coding for metal transporters such as Pmr1, Pmc1, and Vcx1 (Chow et al., 2017; Kmetzsch et al., 2010). Pmc1 seems to play a minor role in ROS defense as well, as a *C. neoformans pmc1* null mutant had decreased expression of several oxidative stress response genes, including *SOD2* (Squizani et al., 2018). In addition, adequate manganese homeostasis would imply a proper balance of ROS defense, as the addition of Mn^{2+} decreases the SOD1 levels in WT cells and alleviates the toxic effect of paraquat in cells lacking *zip3*. Taken together, these results suggest a role in oxidative defense for Zip3, possibly mediated by manganese, as it is a redox active metal with rapid quenching capacity of peroxyl radicals (Coassin et al., 1992).

As ROS production is one of the main strategies macrophages utilize to deal with intracellular pathogens, we sought to determine if Zip3 is important for *C. gattii* survival and replication inside these cells. We did not observe a significant difference in the intracellular proliferation rates between the studied strains. The presence of increased ROS levels itself is not a determinant of lower IPR in macrophages, as such host cells use mainly reactive nitrogen species (RNS) to kill cryptococci (TOHYAMA et al., 2007). It is also reasonable to speculate that the alterations in manganese levels would alter the proliferation and even survival inside macrophages. However, fungal cells suffer from manganese deprivation in the infection milieu (Crawford and Wilson, 2015). In this way, similar IPR of WT and cells lacking *ZIP3* may be due to possible compensatory effects of other genes or that the defects in the manganese levels plays a minor effect of survival and replication inside phagocytes. However, a striking observation was the lower fungal loads of the *ZIP3* null mutant cells. No difference was observed in the polysaccharide capsule size or chitin oligomer distribution, which are important regulators of phagocytosis by host cells (Leopold Wager et al., 2016; Ost et al., 2017). However, the *zip3* null strain secreted more GXM into the supernatant. This would explain the lower fungal loads observed in macrophages co-incubated with these cells, as GXM is the major component of the capsule and has several immunoregulatory effects on host cells, and antiphagocytic properties (Ellerbroek et al., 2005; Zaragoza et al., 2009).

To evaluate the importance of Zip3 in virulence, we infected larvae of the insect model *G. mellonella.* These larvae have a similar innate immune system to that of vertebrates and support temperatures up to 37 °C, and are largely utilized in virulence and antibiotic drug studies in vivo (Firacative et al., 2014; Mylonakis et al., 2005). Cryptococcal hypervirulent strains are commonly associated with higher phagocytosis by macrophages, a phenotype more associated with virulence than high intracellular division itself (Bojarczuk et al., 2016; Mansour et al., 2011). Also, the chemical inhibition of phagocytosis in the *G. mellonella* model led to decreased virulence of this pathogen (Lim et al., 2018).

To further investigate the impact of Zip3 in cryptococcal virulence, we analyzed some virulence determinants. GXM, a key cryptococcal virulence factor, is synthesized in the Golgi (Yoneda and Doering, 2006). Considering the potential association between Zip3 and the Golgi, alterations in the synthesis of this polysaccharide would be expected. In fact, we observed higher GXM secretion in the *zip3* mutant, which also produced larger EVs. GXM secretion requires vesicular export to the extracellular space (Rodrigues et al., 2007), and larger EVs have been recently associated with increased concentrations of extracellular GXM (Reis et al., 2019). Therefore, our present results suggest an association between Golgi physiology, vesicular GXM export, and Zip3 functionality.

Based on the altered profile of GXM secretion in the *zip3* mutant, we hypothesized that other virulence factors associated with secretory activity could be affected. Indeed, we observed lower melanin production by these cells over time. Melanin is important for the survival of

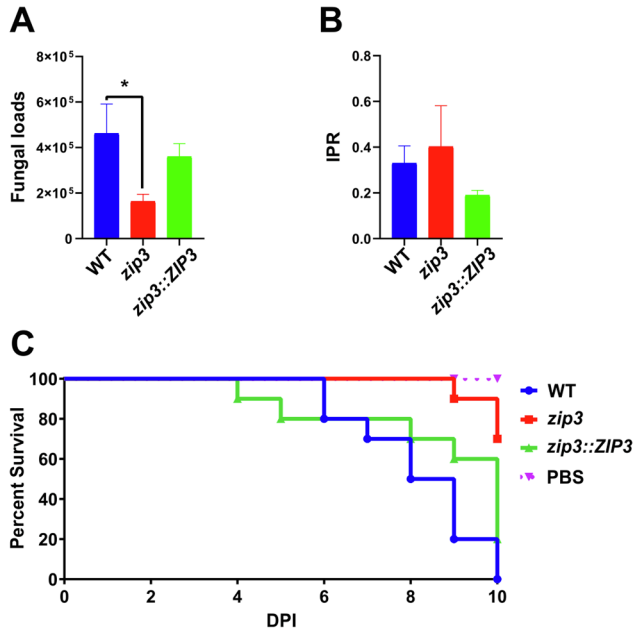

Fig. 5. Lack of Zip3 affects the virulence of *C. gattii*. (A) The fungal loads were determined in host cells after 2 h interaction of *C. gattii* (WT, *zip3*, and *zip3::ZIP3*) cells with PMA-activated J774.16 macrophage-like cells. **(B)** The intracellular proliferation rate (IPR) was determined as the ratio between the fungal loads at 24 h and 2 h incubation with PMA-activated J774.16 cells. Data are the means and SD of three biological replicates. One-way ANOVA followed by Tukey's multi-comparison test was performed. The asterisk denotes statistically significant differences (*$P < 0.05$). **(C)** Survival curve for determining virulence in vivo. Strains were inoculated via the last proleg in *G. mellonella* larvae (n = 10). The larvae were maintained at 37 °C with daily monitoring of mortality for up to 10 days post infection (DPI). Survival curves were analyzed using the log-rank (Mantel–Cox) test.

this yeast in the environment and in the host, as it is capable of stabilizing free radicals, and helps maintain the cell wall structure (Dadachova et al., 2008; Wang and Casadevall, 1994). This result could also explain the higher sensitivity to ROS-generating factors as well as the decreased virulence in vivo.

In conclusion, we provide evidence that Zip3 plays a role in the modulation of cryptococcal virulence: (i) as a predicted Golgi-associated transporter, Zip3 may regulate GXM secretion; (ii) Zip3 is necessary for proper redox homeostasis; (iii) Zip3 also regulates the stress response. Taken together, our results allow us to conclude that Zip3 may regulate several aspects related to virulence in cryptococcal cells. However, the mechanistic details of Golgi association with Zip3, as well as the regulation of virulence determinants secretion due to the activity of this putative metal transporter remains to be elucidated.

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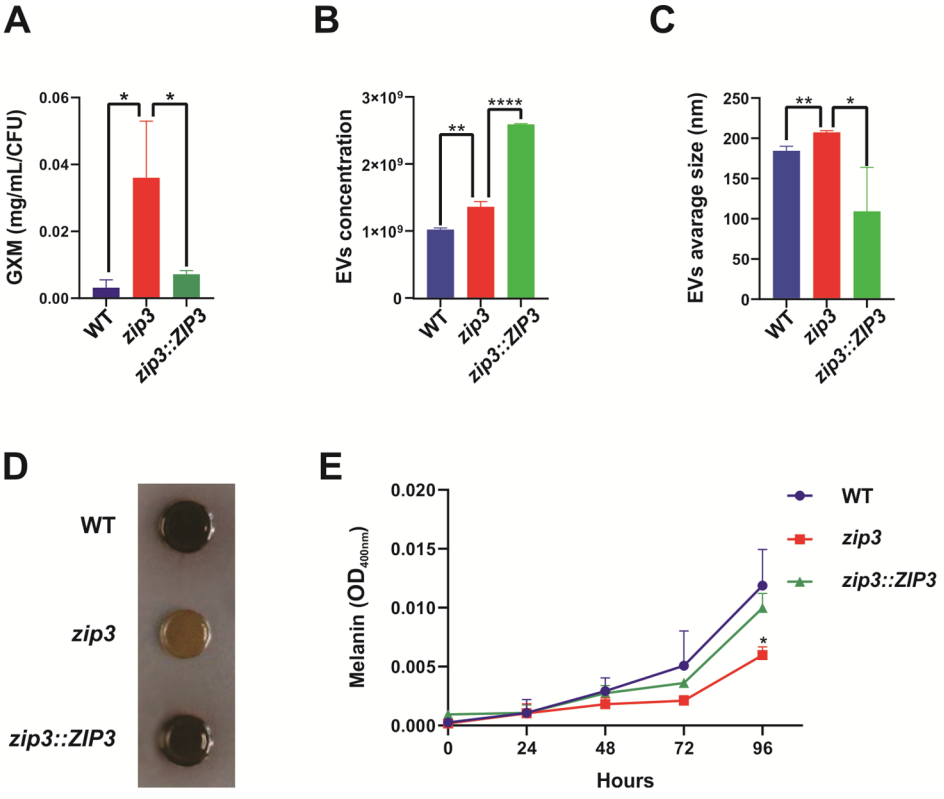

Fig. 6. The effects of *ZIP3* deletion on secreted virulence determinants. (A) ELISA detection of the main capsule component GXM in the supernatant of cryptococcal strains incubated in DMEM at 37 °C for 48 h. The quantification **(B)** and average size **(C)** of EVs recovered from *C. gattii* WT, *zip3* or *zip3::ZIP3* strains. . **(D)** Growth of *C. gattii* strains in minimal medium containing L-DOPA to visualize melanization after 48 h incubation at 30 °C. **(E)** Quantification of secreted melanin in the supernatant of each strain in minimal medium containing L-DOPA. Data are the means and SD of three biological replicates. One-way ANOVA followed by Tukey's multi-comparison test was performed. The asterisks denote statistically significant differences (*$P < 0.05$; **$P < 0.01$).

Appendix A. Supplementary material

Supplementary data to this article can be found online at https://doi.org/10.1016/j.fgb.2020.103438.

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The role of the Nramp transporter of *Cryptococcus gattii* in metal acquisition and virulence

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Abstract

Cryptococcus gattii is one of the etiologic agents of cryptococcosis, a potentially fatal disease that affects humans and animals. The successful infection of mammalian hosts by cryptococcal cells relies on their ability to infect and survive in macrophages. Such phagocytic cells present a hostile environment to intracellular pathogens via the production of reactive nitrogen and oxygen species, as well as low pH and reduced nutrient bioavailability. To overcome the low-metal environment found during infection, fungal pathogens express high-affinity transporters, such as the manganese Nramp transporters. Manganese is essential for life, and in fungi is related to central metabolism, oxidative stress response and Golgi glycosylation. We have previously demonstrated that disruption of manganese homeostasis in *C. gattii* by the deletion of the Golgi transporter Zip3 coding gene led to impaired cell fitness, oxidative misbalance, as well as reduced virulence. Here, we characterize the *SMF1* gene of *C. gattii*, the only Nramp coding gene copy in this fungi genome. Smf1 is predicated to be located in the cell membrane and act in metal acquisition, primarily manganese and iron. Cryptococcal cells lacking Smf1 presented higher sensitivity to the chelating agents EDTA and EGTA, as well as to the ROS generating agent paraquat. Our analysis also suggested that the Nramp copy in Cryptococcus is related to an ancestral protein as it differs in sequence and regulation from those of *Saccharomyces cerevisiae*. Moreover, cells lacking Smf1 displayed a decrease in virulence in a non-vertebrate model of infection.

Keywords: *Cryptococcus gattii*, manganese, Nramp.

1. Introduction

Manganese is a redox-active metal essential for life. In fungi, these ions are associated with a variety of processes including central metabolism, oxidative stress and Golgi's glycosylation of proteins and possibly other molecules (Crowley et al., 2000; Kelliher and Kehl-Fie, 2016). During infection, the host restrain the bioavailability of transition metals from invading microbes as an active response known as nutritional immunity (Hood and Skaar, 2012). Extracellular manganese, as well as zinc, is sequestered by the heterodimer S100A8/S100A9 calprotectin which constitutes virtually half of the protein content on neutrophils (DALE et al., 1983; Damo et al., 2013). The effectiveness of this system has been demonstrated against fungi pathogens such as *Candida albicans*, *Aspergillus fumigatus*, *Aspergillus nidulans* and *Cryptococcus neoformans* both *in vitro* and *in vivo* (Bianchi et al., 2011; Clark et al., 2016; Mambula et al., 2000; Urban et al., 2009). Intracellular pathogens also face a low manganese environment due to the activity of the divalent cation transporter NRAMP1 (SLC11A1), also known as DMT-2. This transporter is continuously expressed by phagocytes and incorporated in the membrane of lysosomes and maturing phagosomes where it acts in the transportation of mainly iron and manganese into the cytosol and away from the pathogen (Gruenheid et al., 1997; Jabado et al., 2000). Loss of activity in this protein is related to increased susceptibility to intracellular pathogens (Bellamy, 1999; Cellier et al., 2007; Li et al., 2011).

In order to overcome this micronutrient starvation imposed by the host, pathogens have evolved a number of strategies, including the production of low-molecular-weight molecules for extracellular metal capture, expression of high-

affinity membrane metal transporters and enzymes capable of utilizing more than one metal as cofactor (Barwinska-Sendra et al., 2020; Kelliher and Kehl-Fie, 2016; Wu et al., 2021). The main manganese acquisition system in fungi cells is represented by the Nramp (natural resistance-associated macrophage protein) family of transporters, a class of metal-ion transporters conserved from bacteria to mammals (Cellier et al., 1995). Nramp transporters are involved in the translocation of a range of divalent metals through membranes towards the cytosol utilizing a proton gradient, including manganese, iron, copper, cadmium and cobalt (Portnoy et al., 2000). Although much is known about manganese mobilization and homeostasis in the model yeast *Saccharomyces cerevisiae*, information regarding fungal pathogens is limited (Culotta et al., 2005; Thines et al., 2019). The model yeast present three Nramp homologues, while Smf1p and Smf2p are associated with manganese homeostasis, Smf3p is associated with iron translocation (Portnoy et al., 2000). Smf1p is a high affinity transporter expressed in the cell membrane when manganese uptake is required, while Smf2p is a low affinity manganese transporter located in Golgi derived vesicles and is responsible for regulating intracellular levels of manganese (Cohen et al., 2000; Portnoy et al., 2000).

Cryptococcus gattii is an etiologic agent of cryptococcosis, a potentially fatal disease. The disease is characterized by a primary lung infection that may disseminate to the whole organism, leading ultimately to meningoencephalitis (Kronstad et al., 2011). *C. gattii* was responsible for the outbreak the pacific northwest in 1999 and is gaining more attention due to its capability to infect immunocompetent hosts, increased virulence and a shift in habitat towards temperate climate zones (Akins and Jian, 2019; Datta et al., 2009). Subnotification

of cases is estimated due to misdiagnosis, largely because the pulmonary disease is easily misled as pulmonary cancer, tuberculosis or bacterial pneumonia (Iverson et al., 2012; Setianingrum et al., 2019; Tintelnot et al., 2015). Despite the best available treatments, the mortality rate of *C. gattii* infections can range up to 33% (Harris et al., 2011).

The successful infection of mammalian hosts by cryptococcal cells relies on their ability to infect and survive in macrophages (Johnston and May, 2013). Our group has previously demonstrated the importance of the ZIP (Zrt, Irt-like protein) family transporters Zip1 and Zip2 in zinc acquisition and virulence in *C. gattii* (Schneider et al., 2015). We have also evaluated the role of the Zip3 transporter involved in manganese translocation in the Golgi. Disruption of its coding gene led to increased manganese tolerance, higher reactive oxygen species sensitivity, and altered production of some virulence traits, such as melanin deposition, secretion of glucuronoxylomannan, ultimately impacting virulence itself in an insect model of infection (Garcia et al., 2020). The only *C. neoformans* Nramp member has been previously shown to integrate in the cell membrane and to be capable of translocating manganese, iron, cobalt and nickel (Agranoff et al., 2005). In order to further elucidate the manganese homeostasis in this pathogen, we present a functional characterization of the *SMF1* gene of *C. gattii*, a member of the Nramp family of transporters, and its impact on metal acquisition and virulence.

2. Materials and methods

2.1 Bioinformatic analyses

Protein sequences were retrieved from FungiDB database (Basenko et al., 2018) using as query the presence of the Nramp conserved domain (PFAM code PF01566). Prediction of transmembrane helices were performed using the TMHMM (Krogh et al., 2001) platform in FungiDB. The amino acid alignment was built using PRANK v.100701, without manual curation (Löytynoja and Goldman, 2010). The best-fit evolutionary model (LG+I+G) was estimated using Prottest 3.4 (Darriba et al., 2011). Phylogenetic reconstruction (Maximum Likelihood) was conducted using PhyML 3.1 (Guindon et al., 2010) with aLRT SH-like (approximate likelihood ratio test Shimodaira–Hasegawa) branch support estimation (Anisimova et al., 2011; Anisimova and Gascuel, 2006).

2.2 Strains and culture conditions

Cryptococcus gattii R265 was used in this work. Fungal strains were routinely cultured in YPD medium (2% glucose, 2% peptone, and 1% yeast extract) incubated at 30°C on a constant rotation platform at 200 rpm. Agar was added at a final concentration of 1.5% when solid media was used. YNB (yeast nitrogen base) without amino acids, supplemented with 2% glucose and asparagine (40 mM), was used for the phenotypic assays. The low-manganese medium (LMM, pH 7.4) contained (per liter; all obtained from Sigma): glucose (G5767, 5 g), dipotassium monohydrogen phosphate (P3786, 0.4 g), asparagine (A0884, 5 g), calcium chloride dihydrate (C3881, 0.25 g), thiamine (T4625, 0.4 mg), cupric sulfate pentahydrate (C6283, 0.005 mg), zinc sulfate heptahydrate (Z0501, 2 mg), magnesium sulfate heptahydrate (M9397, 80 mg), sodium molybdate (MI003, 0.46 mg), boric acid

(B0394, 0.057 mg) and iron sulfate heptahydrate (F7002 , 2 mg), with the addition of manganese sulfate heptahydrate (M2773) when necessary.

2.3 Null strain construction

The split marker methodology (Jung et al., 2018) was used to construct de *SMF1* mutant strains. The 5' and 3' flanking regions (~1000 bp) of the *SMF1* gene (CNBG_6162) were PCR-amplified and gel-purified using a Illustra GFX PCR DNA and Gel Band Purification Kit (GE Healthcare). The *HYG* marker was amplified from the pJAF15 plasmid (Fraser et al., 2003) and purified by the same methodology. Double joint-PCR was utilized to amplify the 5'-split and 3'-split gene disruption cassettes. Biolistic transformation was performed to introduce the deletion cassettes, about 3 µg each, in *C. gattii*. The mutant strains were selected in solid media containing 150 µg/mL of hygromycin B and confirmed by semi-quantitative reverse transcription (RT)-PCR. Supplementary Table 1 lists the primers used for cassette construction and screening.

2.4 RT-qPCR analysis

For RNA extraction, the *C. gattii* R265 strain was pre-inoculated in YPD medium overnight. The cells were then washed three times with phosphate-buffered saline (PBS 1X) and counted in a Neubauer chamber. A total of 10^7 cells were inoculated in LMM medium (control) or LMM medium added of a range of $MnSO_4$ concentrations, up to 50µM. The cells were incubated for 2 h at 30°C. RNA was isolated using TRIzol (Invitrogen) after cell lysis with liquid nitrogen using a mortar and pestle. RNA integrity and concentration were evaluated by electrophoresis on 1% agarose gel and by fluorometry using a Qubit fluorometer and Quant-iT RNA Assay Kit according to the manufacturer's instructions (Invitrogen). Complementary

DNAs (cDNAs) were prepared using DNase (Promega)-treated total RNA samples (1000 ng) with ImProm-II Reverse Transcriptase (Promega) using oligo-dT. Quantitative RT-PCR (qRT-PCR) was performed on a StepOne Real-Time PCR System (Applied Biosystems) with thermal cycling conditions set with an initial step at 95°C for 10 min, followed by 40 cycles at 95°C for 15 s, 60°C for 15 s, and 72°C for 60 s. The PCR were conducted at a final volume of 20 µL, containing 2 µL cDNA (2.5 ng/µL), 2 µL SYBR Green (1:1000, Invitrogen), 0.1 µL dNTP (5 mM), 2 µL PCR buffer 10×, 1.2 µL $MgCl_2$, 0.05 U Platinum Taq DNA Polymerase (Invitrogen), and 0.2 µL each primer (5 pmol). Melting curve analysis was performed at the end of the reaction to confirm the presence of a single PCR product. All experiments were performed using three independent cultures, and each cDNA sample was analyzed in triplicate for each primer pair. Data were normalized to actin cDNA amplified in each set of PCR experiments. Relative expression was determined by the comparative threshold cycle ($2^{-\Delta\Delta CT}$) method (Schmittgen and Livak, 2008). Supplementary Table 1 lists the primers used for quantifying each cDNA level.

2.5 Spot plate assay

The wild-type (WT) and mutant strains *smf1.6* and *smf1.28* were cultivated in YPD medium overnight at 30°C under agitation (200 rpm) and washed three times with PBS. A suspension of cells (OD_{600} = 1.0) from each strain was prepared and serially diluted up to 10,000×. A 3 µL aliquot from each suspension was spotted on YNB medium or YNB medium containing 0,5 mM EDTA (Ethylenediamine tetraacetic acid), 10 mM EGTA (ethylene glycol-bis(β-aminoethyl ether)- tetraacetic acid), 5 mM manganese sulfate, or 1mM paraquat. A 3 µL aliquot of each

suspension was also spotted on LMM or LMM added of a range of manganese or iron sulfate. The plates were incubated at 30°C for 2 days and photographed.

2.6 Growth assays

After overnight culture of WT and *smf1* null mutant strains in YPD medium at 30°C, the cells were collected by centrifugation and washed three times with PBS. The cell density was determined using a Neubauer chamber. A total of 20,000 cells were suspended in 100 µL YNB and distributed into 96-well plates. An additional 100 µL YNB (control) or YNB supplemented with increasing concentrations of EDTA (final concentration 0.5 mM), EGTA (final concentration 5 mM), or paraquat (final concentration 0.125 mM) were also added. The plates were incubated for 24 h at 30°C or 37°C, and the OD_{600} was determined using a SpectraMax i3 microplate reader.

2.7 Virulence assay in *Galleria mellonella* larvae

Galleria mellonella larvae (15–20 mm and 220–260 mg, n = 10) with no signs of infection, such as the absence of grey pigmentation, were infected by injection into the last proleg with 10 µL 10^5 cells from the WT, or *smf1* null mutant strains suspensions previously cultured in YPD medium on a shaking platform (30 °C). The same volume of sterile PBS or dimethyl sulfoxide (DMSO) was used as negative and positive controls, respectively. All larvae were placed in sterile Petri dishes and maintained at 37°C with daily monitoring of mortality for up to 10 days.

2.8 Statistical analysis

Statistical differences were analyzed using one-way analysis of variance (ANOVA). All values reported as means and the standard error (SD). Survival curves were analyzed using log-rank (Mantel-Cox) test.

3. Results

3.1 *In silico* characterization of *C. gattii* Smf1

In order to gain insights about functions associated with the only Nramp associated product in *C. gattii,* a set of *in silico* predictions were performed. The similarity between the sequence of *C. gattii* Nramp Smf1 and *S. cerevisiae* Nramp Smf1p and Smf2p were 32% with 98% sequence coverage and 40% with 73% sequence coverage, respectively. The *C. gattii* copy was predicted to present 11 transmembrane domains, which is a standard for fungi Nramp associated proteins, as revealed by TMHMM and InterProScan analysis in FungiDB. Interestingly, the cryptococcal copies presented two predicted Nramp domains (PFAM accession PF01566), while the *S. cerevisiae* orthologs present only one (Figure 1A). Moreover, *C. gattii* Smf1 was predicted to localize to the cytoplasm (49.8%) and plasma membrane (49.6%), the same pattern was observed for *S. cerevisiae* Smf1p with 49.6% and 49.5% respectively, according to YLoc prediction Server (Briesemeister et al., 2010). Both sequences were also predicted to localize to endomembrane system or plasma membrane with scores of about 90% and 50%, respectively, according to the BUSCA prediction server (Savojardo et al., 2018). A phylogenetic analysis utilizing the Nramp domain-containing sequences from *S. cerevisiae* and important fungal pathogens *C. gattii, C. neoformans, A. fumigatus* and *C. albicans* was performed, as some members of this set of proteins have been functionally characterized (Figure 1B). The saccharomycete sequences grouped together and a separation between three distinct clusters can be observed. While the cryptococcal

Nramp domain-containing sequences seem to be related to an ancestral Nramp related protein.

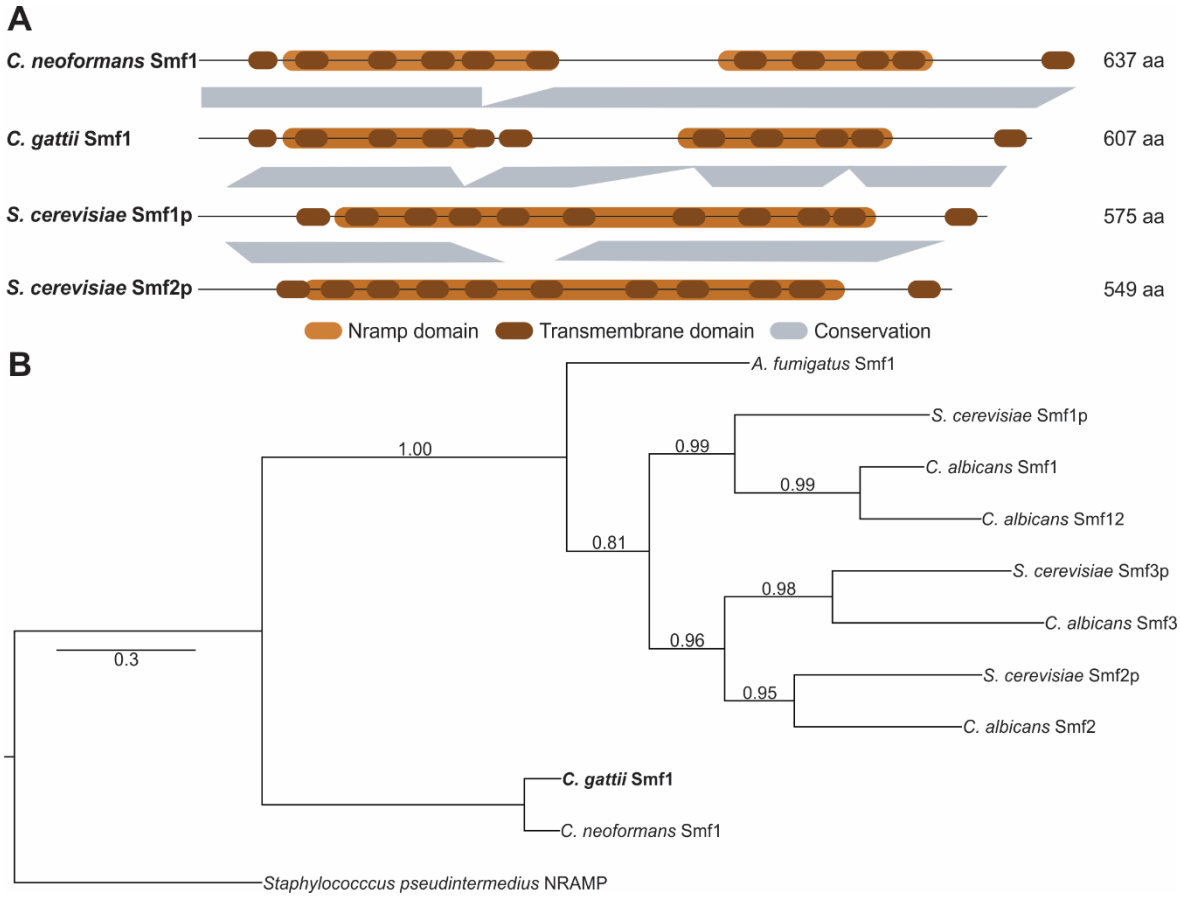

Figure 1: *In silico* analysis of *C. gattii* Smf1. (A) Comparison between the Nramp-containing sequences from *C. gattii*, *C. neoformans* and *S. cerevisiae*. The Nramp domains are shown in orange, transmembrane domains are shown in brown and identity segments are shown as a grey box; identity is always shown against *C. gattii* sequence. The length of each protein sequence, in amino acids, is shown to the right. **(B)** Phylogenetic analysis applying the ML method including Nramp sequences from *C. gattii* (Smf1 – CNBG_6162), *C. neoformans* (Smf1 - CNAG_5640), *A. fumigatus* (Smf1 – Afu4g10990), *C. albicans* (Smf1 – C2_07160W_B; Smf12 – C4_00990W_A; Smf2 – C1_13840W_A; Smf3 – C2_00580C_A) and *S. cerevisiae* (Smf1 – YOL122C; Smf2 – YHR050W; Smf3 – YLR034C). A Nramp ortholog from *Staphyloccoccus pseudintermedius* was used as outgroup. Only branches with high confidence of bootstrap (> 80%) are annotated. A bar marker indicates the genetic distance, which is proportional to the number of amino acids substitutions.

3.2 Participation of Smf1 in metal acquisition

The only *C. neoformans* Nramp orthologue has been shown to locate to the cell membrane and to be able to transport manganese, iron, cobalt and nickel in heterologous expression models (Agranoff et al., 2005). The similarity between Nramp sequences from *C. neoformans* and *C. gattii* are about 84% with 100% sequence coverage. In order to elucidate the participation of *C. gattii* Smf1 in metal acquisition, a series of solid medium growth assays was performed in null mutants strains (*smf1.6* and *smf1.28*) of the *SMF1* gene. We evaluated their sensitivity to metal chelating agents such as EDTA and EGTA, which present lower affinity for magnesium, making it more selective for calcium and manganese ions. The tolerance to high manganese concentrations and sensitivity to the oxidative agent paraquat was also evaluated. Finally, we made use of a low-manganese medium (LMM) supplemented with a range of iron and/or manganese concentrations, but we could not observe any differences in the growth capabilities of the *smf1* null mutants compared to the WT in any of the conditions evaluated (Figure 2).

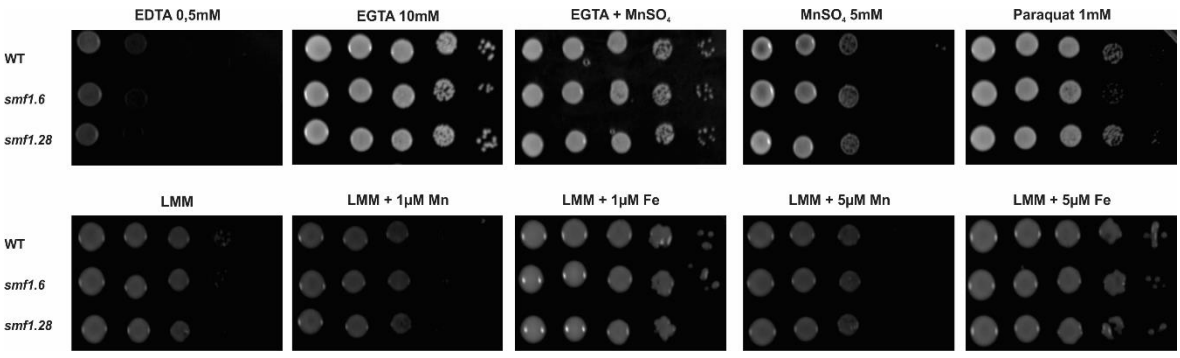

Figure 2: Spot plate assay. A suspension of cells (OD_{600} = 1.0) from each strain was prepared and serially diluted up to 10,000×. A 3 µL aliquot of each suspension was spotted on YNB medium or YNB medium containing the said concentrations of the chelating agents EDTA and EGTA, or $MnSO_4$ or the oxidative agent paraquat (upper row). A 3 µL aliquot of each suspension was spotted on Low-manganese medium (LMM) or LMM added of the said concentrations of manganese or iron salts. The plates were incubated 30°C for 2 days and photographed.

In the yeast model *S. cerevisiae* the lack of the cell membrane ortholog Smf1p resulted in increased sensitivity to EGTA, which could be alleviated with the addition of low concentrations of manganese or copper (Cohen et al., 2000). The absence of Smf2p also led to increased sensitivity towards EGTA, and the double null mutant presented even higher sensitivity. The *SMF1* null mutant strain also presented higher tolerance to manganese and cobalt (Cohen et al., 2000). Interestingly, null mutants for *SMF1* presented no alteration in intracellular manganese levels compared to WT cells, and no defect in manganese dependent enzymes could be observed (Luk and Culotta, 2001; Supek et al., 1996). On the other hand, the manganese supplied by Smf2p is bioavailable and utilized by manganese-requiring enzymes, such as Sod2p (superoxide dismutase) (Luk and Culotta, 2001). Loss of Smf2p has a profound impact on manganese accumulation and bioavailability, even under manganese surplus conditions, in contrast to loss of Smf1p.

However, cryptococcal cells present only one Nramp ortholog that is predicted to locate to the cell membrane and act as a high affinity transporter, maybe fulfilling the roles of all *S. cerevisiae* orthologs. We evaluated the expression level of *SMF1* gene of *C. gattii* in response to increasing manganese concentrations. We could observe a significant reduction of expression in the conditions of 10 and 50 µM, which characterize metal sufficient conditions (Figure 3A). This pattern of expression is recurrent among high affinity transporters that are only expressed when the cell metal levels are below optimal. We also evaluated the growth of the null mutant strains against the metal chelators EDTA and EGTA in liquid medium,

which consist of a more robust assay. *C. gattii* cells lacking *SMF1* product displayed increased sensitivity to both EDTA (Figure 3B) and EGTA (Figure 3C).

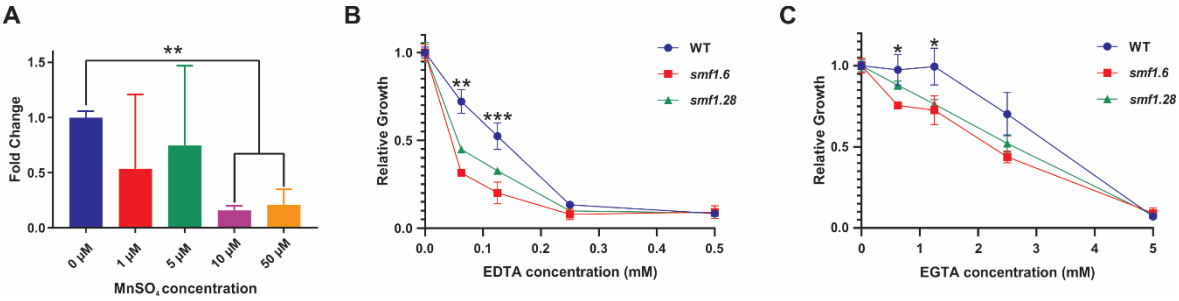

Figure 3: Smf1 participates in metal homeostasis. (A) RT-qPCR analysis of the Nramp transporter coding gene SMF1. A total of 10^7 cells from WT strain was inoculated in LMM medium (control) or in LMM containing a range of $MnSO_4$ concentrations for 2 h at 30°C. The measured quantity of the mRNA in each sample was normalized using the Ct values obtained for the *ACT1* gene. Data are the means and SD of three biological replicates. **(B)** Measurement of WT, *smf1.6* and *smf1.28* strains growth after 24h at 30°C in YNB medium (control) or YNB added of a range of EDTA concentrations by OD_{600} determination. **(C)** Measurement of WT, *smf1.6* and *smf1.28* strains growth after 24h at 30°C in YNB medium (control) or YNB added of a range of EGTA concentrations by OD_{600} determination. Two-way ANOVA followed by Tukey's multi-comparison test was performed. The asterisks denote statistically significant differences (*$P < 0.05$; **$P < 0.01$; ***$P < 0.001$).

3.3 Full cryptococcal virulence is compromised in the absence of Smf1

Manganese plays a major role in redox balance. Enzymatically, as cofactor of SOD2 in the mitochondrial matrix and in antioxidant non-protein complexes containing lactate or phosphate (Barnese et al., 2012, 2008). In *S. cerevisiae*, Smf1p seems important in this context, supplying the cell for non-protein manganese complexes, as the addition of manganese salts to cells lacking proper redox defenses is able to reverse all phenotypes observed, and this rescue is dependent on the cell membrane transporter (Luk and Culotta, 2001). We observed a slight increase in sensitivity of *C. gattii* null mutant strains to the oxidative agent paraquat

in liquid medium (Figure 4A). This higher sensitivity can be due to the conserved function of supplying manganese for antioxidant complexes in the cell.

As disruption of manganese homeostasis has been shown to impact virulence in several pathogens, including *C. gattii* (Garcia et al., 2020), we sought to evaluate the effect of the absence of *SMF1* in a non-mammalian model of Cryptococcosis. Infection of *G. mellonella* larvae with WT caused death in 50% of the larvae (LT_{50}) in 6.6 days. However, it took 10 days for 50% of the larvae infected with the null *SMF1* mutant strains to die (Figure 4B). The difference in virulence compared to the WT was of (P=0.0237) for the *smf1.6* strain and (P=0.0129) for the *smf1.28* strain.

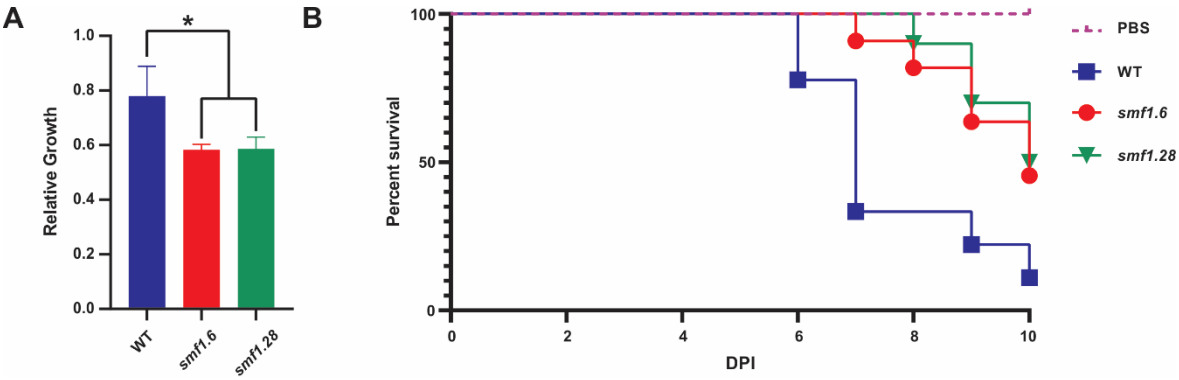

Figure 2: Lack of Smf1 affects the virulence of *C. gattii*. (A) Measurement of WT, *smf1.6* and *smf1.28* strains growth after 24h at 30°C in YNB medium (control) or YNB added 0.25 mM of the oxidative agent Paraquat by OD_{600} determination. Two-way ANOVA followed by Tukey's multi-comparison test was performed. The asterisks denote statistically significant differences (*$P < 0.05$). **(B)** Survival curve for determining virulence in vivo. A total of 10^5 cells from each strain were inoculated via the last proleg in *G. mellonella* larvae (n = 10). The larvae were maintained at 37°C with daily monitoring of mortality for up to 10 days post infection (DPI). Survival curves were analyzed using the log-rank (Mantel–Cox) test.

4. Discussion

The acquisition of manganese by fungal cells is carried mainly by the Nramp family of transporters. *S. cerevisiae* present three homologues of Nramp

transporters, but only Smf1p and Smf2p are associated with manganese homeostasis (Portnoy et al., 2000). Homologues of *S. cerevisiae* SMFs are present in the majority of fungal genomes, with exceptions being the plant pathogens *Magnaporthe grisea* and *Alternaria brassicicola* (Diss et al., 2011). When present, the number of copies of SMF-related genes vary between one and five per genome. The Saccharomycete class probably underwent duplication and specialization events, as the SMFs paralogs of *S. cerevisiae* have been shown to perform alternative functions (Cohen et al., 2000; Portnoy et al., 2000). However, the cryptococcus species complex present only one copy of Nramp-related gene and our analysis suggests its closer to an ancestral protein.

The presence of a single SMF representative can raise questions about function and specificity towards the substrate, as *S. cerevisiae* Smf1p and Smf2p transport manganese and iron from the extracellular compartment and intracellular vesicles, respectively, and Smf3p transports exclusively iron from the vacuole (Cohen et al., 2000; Portnoy et al., 2000). The Nramp orthologue of *C. neoformans*, which present high similarity to *C. gattii* orthologue, was characterized as a pH-dependent transporter of manganese, iron, cobalt and nickel and to localize to the cell membrane in a heterologous model of expression (Agranoff et al., 2005). Hypothetically, the single Nramp copy can perform the functions of all *S. cerevisiae* orthologues, or act in a similar fashion as Smf1p, due to its location and substrate specificity, and designate intracellular manganese supply to other manganese transporters, such as the Golgi Zip3 (Garcia et al., 2020).

There is a requirement for trace metals in order to maintain cell fitness. Iron is required for oxidoreductase enzymes, copper is a component of laccase, an

established cryptococcal virulence determinant (Williamson, 1997), and the Cu/Zn superoxide dismutase (Narasipura et al., 2003). Manganese is required for a series of biological processes, including the mitochondrial isoform of superoxide dismutase and the Golgi sugar transferases involved in protein processing (Agaphonov et al., 2007; Bates et al., 2005; Narasipura et al., 2005; Navarro-Arias et al., 2016). The host, however, utilizes a series of approaches in order to restrain this essential trace metals from pathogens. The role of manganese in host defense against bacteria, and how bacteria acquire and utilize this metal in the infection site is now better understood (Kehl-Fie and Skaar, 2010; Kelliher and Kehl-Fie, 2016; Morey et al., 2015; Wu et al., 2021). However, information about manganese homeostasis and regulation is still missing for classic fungal pathogens.

A decrease in the expression of *C. gattii* Smf1 in relatively low manganese concentrations at a transcriptional level was observed. This could also be part of an ancestral expression system, as *S. cerevisiae* SMFs are regulated only at a post-translational level by protein turnover and localization shift in response to manganese concentrations (Jensen et al., 2009; Liu and Culotta, 1999). These transporters are continuously expressed in *S. cerevisiae* and present no alteration in mRNA levels regardless of manganese concentration, being usually targeted for degradation in vacuoles. In manganese starvation, however, the amount of Nramp transporters increases as Smf1p is directed to the cell membrane and Smf2p to intracellular vesicles in order to function. A similar condition was observed for the white-rot fungus *Phanerochaete sordida* Smf homologue, as no alteration in mRNA levels were detected with the addition of up to 1mM of manganese (Mori et al., 2018).

A defect in growth of *C. gattii* cells lacking Smf1 could be observed in the presence of chelating agents, such as EDTA and EGTA, similarly to *S. cerevisiae* model (Cohen et al., 2000). However, we could not observe the same phenotype in solid medium. This can be the result of activity of other transporters that can also transport manganese, like the high affinity phosphate transporter Pho84, that is associated with manganese acquisition in the absence of Nramp transporters (Jensen et al., 2003). Disruption of *DmtA* in *Aspergillus niger*, which presents a high sequence identity to Smf1p and Smf2p, eliminated the acquisition of manganese at low extracellular concentrations, and led to reduced intake at high concentrations. Moreover, absence of *DmtA* also impacted germination and hyphal morphology in this fungi (Fejes et al., 2020).

Besides the sequestration of nutrients, other classical strategies employed by the host macrophages to defend against invading pathogens include acidic pH, and production of ROS and nitrogen reactive species (Gibson and Johnston, 2015; Underhill and Goodridge, 2012). The lower pH, however, is favorable to fungi development and function of Nramp transporters, that rely on a proton gradient in order to transport metals (Agranoff et al., 2005; Levitz et al., 1999). Manganese is a unique redox active metal, in a sense that it always presents antioxidant activity, even in high concentrations, in contrast with other transition metals. Non-protein complexes of manganese can act in the removal of reactive oxygen species (ROS), such as complexes containing lactate and phosphate (Barnese et al., 2012, 2008). Also, several fungal pathogens, including *C. neoformans* and *C. gattii* express a manganese dependent isoform of superoxide dismutase as a strategy against the oxidative burst of phagocytes (Fréalle et al., 2005; Narasipura et al., 2005). *C. gattii*

cells lacking Smf1 presented higher sensitivity to the oxidative agent paraquat than wild-type cells. Smf1p in *S. cerevisiae* has been shown to supply the cell with manganese for non-protein antioxidant complexes (Luk and Culotta, 2001), so this increased sensitivity in *C. gattii SMF1* null mutant strains can be due to a conserved function. Disruption of the manganese Golgi transporter Zip3 in *C. gattii* also resulted in redox disequilibrium with higher sensitivity towards oxidative agents and drastic change in expression of classic redox defense systems in the cell (Garcia et al., 2020).

As metal acquisition is important in the scarce condition of host infection and ROS production is one of the main strategies macrophages utilize to deal with intracellular pathogens, we sought to determine if Smf1 is important for *C. gattii* virulence utilizing an insect model. *G. mellonella* larvae have a similar innate immune system to that of vertebrates and support temperatures up to 37°C, and are largely utilized in virulence and antibiotic drug studies in vivo (Firacative et al., 2014; Mylonakis et al., 2005). We observed a decrease in virulence of cells lacking Smf1 compared to wild-type. *SMF1* coding gene has also been shown to be highly expressed in a murine model of infection, corroborating with its role in virulence (Ferrareze et al., 2017). Probably the most important condition for the activity of this transporter is during infection process where high affinity transporters are needed in order to overcome the metal starvation imposed by the host. Bacterial Nramp orthologues are widely responsible for metal acquisition and resistance to ROS and acidic pH during infection, playing a major role for the virulence of several bacterial species (Anderson et al., 2009; Boyer et al., 2002; Champion et al., 2011; Colomer-Winter et al., 2018; Handke et al., 2018; Perry et al., 2012; Shabayek et al., 2016).

In conclusion, we provide some evidence that Smf1, the only Nramp-related gene for *C. gattii*, is important for metal acquisition under scarcity metal conditions, as well as contributes to redox equilibrium and virulence in this pathogen.

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6. Supplementary information

Supplementary table 1

Primer	Sequence
qRT F	CCCAAGTGGGTGACTGTATTAG
qRT R	CCAAGACCTAGCTCCACAATC
M13 F KO	gTAAAACGACGGCCAGTG
M13 R KO	CAGGAAACAGCTATGACCATG
HYG-SM1	CGAAGAATCTCGTGCTTTC
HYG-SM2	ATTGACCGATTCCTTGCG
SMF1-5' F	TCTTCAATGCACTTAATCAATCG
SMF1-5' R	CACTGGCCGTCGTTTTAcGGCGGCGTTTACAGGTTAC
SMF1-3' F	CATGGTCATAGCTGTTTCCTGCATTGAGCTGTTGTTTGAGGAG
SMF1-3' R	TCCACTTCGATTCATTCTTCC

4. DISCUSSÃO GERAL

Embora *C. neoformans* afete, principalmente, indivíduos imunocomprometidos e esteja mais relacionado a casos de meningite criptocócica, *C. gattii* é capaz de infectar indivíduos imunocompetentes e está mais relacionado ao quadro pulmonar da doença (KWON-CHUNG *et al.*, 2014). Na criptococose, os macrófagos alveolares são células fundamentais na contenção inicial da doença, visto que são as primeiras células a interagir com os patógenos e sua depleção em diversos modelos de infecção foram relacionados a piores prognósticos da doença (BOJARCZUK *et al.*, 2016; OSTERHOLZER *et al.*, 2009b; SHAO *et al.*, 2005). Dentre as diversas estratégias utilizadas por essas células para combater microrganismos invasores se destaca a imunidade nutricional, que consiste no controle ativo da biodisponibilidade de nutrientes no sítio de infecção (HOOD; SKAAR, 2012). Enquanto se tem um corpo maior de conhecimento a respeito da privação de manganês e mecanismos relacionados a aquisição, transporte e uso desse metal por bactérias durante a infecção, há pouca informação disponível em relação a patógenos fúngicos (KEHL-FIE; SKAAR, 2010; KELLIHER; KEHL-FIE, 2016; MOREY; MCDEVITT; KEHL-FIE, 2015; WU *et al.*, 2021).

O hospedeiro dispõe de dois mecanismos principais para sequestrar manganês do patógeno: um intracelular na forma do transportador NRAMP1 e um extracelular na forma do heterodímero calprotectina S100A8/S100A9 (DAMO *et al.*, 2013; GRUENHEID *et al.*, 1997). Ambos os mecanismos citados já foram descritos como efetivos contra patógenos fúngicos (BIANCHI *et al.*, 2011; CLARK *et al.*, 2016; MAMBULA *et al.*, 2000; PULITI *et al.*, 1995; URBAN *et al.*, 2009). *C. gattii* é descrito como um patógeno intracelular facultativo, visto que é capaz de sobreviver e proliferar dentro de macrófagos (JOHNSTON; MAY, 2013). Para tal, essas leveduras dispõem de um arsenal de determinantes de virulência que auxiliam a sobreviver as estratégias de eliminação dos macrófagos, como pH ácido, restrição e nutrientes e produção de espécies reativas de oxigênio (GIBSON; JOHNSTON, 2015). Para expandir o corpo de conhecimento em relação ao transporte e aquisição desse metal no patógeno fúngico *C. gattii* nós caracterizamos

funcionalmente dois sistemas de importação desse metal nessa levedura, o membro da família ZIP presente no Golgi Zip3 e o membro da família Nramp com localização predita na membrana plasmática Smf1.

Na levedura modelo *S. cerevisiae* o transportador Smf1p é considerado um transportador de alta afinidade por manganês presente na membrana plasmática, porém a sua deleção não afeta drasticamente os níveis desse metal nem a atividade enzimática dependente de manganês na célula (LUK, E. E. C.; CULOTTA, 2001; SUPEK *et al.*, 1996). No entanto, fenótipos severos relacionados a ausência de manganês foram observados na ausência do transportador presente em vesículas intracelulares Smf2p (LUK, E. E. C.; CULOTTA, 2001). *C. gattii*, diferentemente de *S. cerevisiae*, possui somente uma cópia relacionado a família de transportadores Nramp em seu genoma e nossa análise sugeriu que está mais relacionada a uma proteína ancestral do que a um dos parálogos de *S. cerevisiae*. A única cópia presente em *C. neoformans*, que apresenta alta identidade a de *C. gattii*, foi descrita como capaz de transportar manganês e ferro e se localizar na membrana plasmática em modelos de expressão heteróloga (AGRANOFF *et al.*, 2005). Assim sendo, o único transportador Nramp de *C. gattii* pode ser capaz de cumprir as funções relacionadas as três cópias encontradas em *S. cerevisiae*, ou ainda exercer uma função relacionada ao transportador Smf1p de *S. cerevisiae*, devido à similaridade de afinidade por substrato e localização celular, delegando o controle intracelular do metal para outros transportadores, como o Zip3.

Na ausência de Smf1, células de *C. gattii* foram mais sensíveis a presença de agentes quelantes. O mesmo fenótipo foi observado para os transportadores Nramp de *S. cerevisiae*, tornando-se mais severo para os mutantes nulos duplos ou triplo (COHEN, A.; NELSON; NELSON, 2000). Embora a grande maioria dos transportadores Nramp de *S. cerevisiae* seja endereçada para degradação em condições regulares de metais, seus níveis aumentam drasticamente na privação de manganês, quando somente um transportador de alta afinidade que é capaz de capturar seu substrato em concentrações ínfimas do mesmo, seria capaz de promover a aquisição do metal (JENSEN *et al.*, 2009). Ainda que não se saiba a concentração de metais no fagolisossomo, estima-se que seja muito baixa devido a atividade do transportador NRAMP1 (CANONNE-HERGAUX *et al.*, 1999; VIDAL

et al., 1995). Dessa forma, diversas bactérias patogênicas dependem dos seus ortológos Nramp para adquirir manganês durante a infecção e resistir ao pH ácido, presença de ROS e privação de nutrientes impostas pelo hospedeiro (ANDERSON *et al.*, 2009; BOYER *et al.*, 2002; CHAMPION *et al.*, 2011; COLOMER-WINTER *et al.*, 2018; HANDKE *et al.*, 2018; PERRY *et al.*, 2012; SHABAYEK *et al.*, 2016). Portanto, fenótipos severos não são observados devido a falta de Smf1 em *C. gattii* em condições relativamente brandas*,* porém, a aquisição de metais fica prejudicada na presença de agentes quelantes fortes ou durante o processo de infecção. Outro fator que corrobora com essa observação é de que o baixo pH encontrado no fagolisossomo é benéfico para a atividade de Smf1 (AGRANOFF *et al.*, 2005). Ainda, o gene codificante *SMF1* apresentou níveis elevados de expressão na condição de infecção em modelo murino (FERRAREZE *et al.*, 2017). Assim sendo, a célula é capaz de tolerar a perda de seu transportador Nramp de alta afinidade em condições regulares de cultivo, assim como visto para *S. cerevisiae*, no entanto o desbalanço no controle intracelular do metal afeta o funcionamento da célula (LUK, E. E. C.; CULOTTA, 2001).

De fato, a ausência do transportador intracelular Zip3 parece afetar mais a aptidão celular do que a ausência do transportador da família Nramp. Nós pudemos observar um desbalanço redox considerável em células de *C. gattii* ausentes em Zip3 que apresentaram maior acúmulo de ROS em condições de cultivo, bem como um aumento na sensibilidade a diversos agentes pró-oxidantes. O papel do manganês como metal redox ativo é bem descrito em sistemas biológicos, sendo capaz de auxiliar no combate ao estresse oxidativo tanto enzimaticamente, quanto não enzimaticamente (BARNESE *et al.*, 2008, 2012; FRÉALLE *et al.*, 2005). A isoforma mitocondrial da enzima superóxido dismutase, capaz de converter o ânion superóxido a peróxido de hidrogênio e oxigênio é dependente de manganês e fundamental para o correto funcionamento celular (DIAS *et al.*, 2006). A deleção de SOD2 em *C. gattii* levou a incapacidade de crescimento a temperatura de 37 °C na presença de oxigênio do ar, bem como maior sensibilidade a agentes pró-oxidantes, altas concentrações de sais e restrição de nutrientes, totalizando num fenótipo de avirulência em modelo murino de infecção (NARASIPURA; CHATURVEDI; CHATURVEDI, 2005). Nós observamos um rearranjo das defesas

antioxidantes clássicas da célula na ausência do transportador Zip3, com uma drástica redução na expressão das duas isoformas de superóxido dismutase, porém com um aumento na expressão de genes codificantes de catalases e glutationa peroxidases. Catalases são enzimas antioxidantes que catalisam a decomposição de peróxido de hidrogênio a água e oxigênio, e glutationa peroxidases estão envolvidas na reciclagem da proteína antioxidante glutationa (STAERCK *et al.*, 2017). É possível que esse remodelamento das defesas antioxidantes celulares ocorra como um mecanismo compensatório devido a perturbação da homeostase intracelular de manganês, mudando de defesas dependente desse metal para outros mecanismos. Ainda, o defeito de crescimento observado para a linhagem mutante nula *zip3* na presença de agentes geradores de ROS foi completamente resgatado com a suplementação de sais de manganês ao meio de cultivo.

Nós também observamos um pequeno aumento da sensibilidade a agentes pró-oxidantes na ausência de Smf1. Em *S. cerevisiae*, Smf1p está relacionado ao fornecimento de manganês para complexos antioxidantes não-proteicos, como moléculas contendo fosfato e lactato, dessa forma, podendo sinalizar uma conservação de função entre os transportadores presentes na membrana plasmática (LUK, E. E. C.; CULOTTA, 2001). O controle sobre os níveis de ROS intracelulares é importante para o correto funcionamento celular, mas também durante o processo de infecção, visto que uma das principais estratégias utilizadas pelo hospedeiro para combater patógenos é justamente a produção explosiva de espécies reativas de oxigênio e nitrogênio por fagócitos (GIBSON; JOHNSTON, 2015; UNDERHILL; GOODRIDGE, 2012).

A ausência do transportador intracelular Zip3 também levou a uma remodelação da expressão de outros transportadores celulares, como os responsáveis pelo controle dos níveis intracelulares de cálcio VCX1 e PMC1 presentes no vacúolo (KMETZSCH, Livia *et al.*, 2013; KMETZSCH, Lívia *et al.*, 2010). Na linhagem mutante nula *zip3*, foram detectados níveis reduzidos de expressão desses dois transportadores, que atuam diretamente na homeostase de cálcio e ativação da via de calcineurina, um importante regulador da expressão desses canais de metais e importante na resposta a diversos estresses,

principalmente o térmico, como desenvolvimento a temperatura do hospedeiro (CHOW *et al.*, 2017; LIU, J. *et al.*, 1991). Alterações dos níveis de metais intracelulares levando a alterações numa via central como a via da calcineurina pode explicar, parcialmente, fenótipos observados relacionados ao estresse e virulência. Na presença de suplementação com manganês, a linhagem nula *zip3* apresentou um aumento na expressão de Pmr1, um transportador com função oposta ao Zip3, estando envolvido na translocação de cálcio e manganês para dentro do Golgi. Esse aumento da expressão pode estar relacionado com o papel de Pmr1 na eliminação de níveis tóxicos de manganês através da via exolítica (LAPINSKAS, P J *et al.*, 1995). Ainda, o controle da homeostase de manganês e cálcio parece se sobrepor em partes, devido a diversos transportadores compartilhados entre os dois metais. Inclusive, a redistribuição de manganês para organelas como o Golgi ou para fora da célula pode ocorrer mesmo na ausência de Pmr1p, através da ação do transportador de manganês do reticulo endoplasmático Spf1p e de Smf2p na presença de altas concentrações de cálcio em *S. cerevisiae* (GARCÍA-RODRÍGUEZ *et al.*, 2015). Interessantemente, a adição de manganês ao meio não levou a uma alteração significativa na expressão do gene codificador do transportador Zip3 de *C. gattii*, porém, um aumento de sua expressão foi detectado na presença do quelante DTPA, que possui maior capacidade de quelar metais redox ativos como ferro, manganês e cobre (SCHNEIDER *et al.*, 2015).

Outra função importante do transportador Pmr1 é a de suprir manganês para as transferases de açúcar presentes no Golgi (ANTEBI; FINK, 1992; RUDOLPH *et al.*, 1989). Na ausência de Pmr1, diversos fungos apresentam problemas no correto processamento de proteínas e outras moléculas levando a fenótipos severos. Em *C. albicans*, inativação do seu respectivo ortólogo de Pmr1 levou a um processo de glicosilação prejudicado, culminando em montagem de uma parede celular fraca e virulência severamente atenuada em modelo murino (BATES *et al.*, 2005). De maneira semelhante, a perda do seu ortólogo em *Candida guilliermondii* afetou diversos processos, como crescimento celular e morfologia, formação de biofilme, constituição da parede celular, reconhecimento por células imunes e virulência em modelo murino e inseto de infecção (NAVARRO-ARIAS *et al.*, 2016). Defeitos na montagem da parede celular também foram observados na ausência de seus

respectivos ortólogos de Pmr1 em *Hansenula polymorpha*, *Aspergillus nidulans* e *Botrytis cinerea* (AGAPHONOV *et al.*, 2007; JIANG *et al.*, 2014; PLAZA *et al.*, 2015). Embora não possa ser observada nenhuma diferença no tamanho da cápsula polissacarídica nem na distribuição de oligômeros de quitina na ausência de Zip3 em *C. gattii*, que também está predito como localizando no Golgi, pudemos observar, no entanto, alteração na secreção de diversos determinantes de virulência clássicos nessa levedura. O pigmento melanina que apresenta diversas funções para sobrevivência desse fungo no ambiente e dentro do hospedeiro estava menos presente, ao mesmo passo que o principal constituinte da cápsula polissacarídica GXM foi mais secretado quando comparado a linhagem selvagem. Ainda, foi detectado um perfil alterado de vesículas extracelulares, que sabe-se serem capazes de carregar determinantes de virulência, incluindo GXM e melanina (RODRIGUES *et al.*, 2008, 2007). Essas alterações no perfil de secreção de determinantes de virulência clássicos podem ser responsáveis pela menor taxa de fagocitose observada por macrófagos para a linhagem mutante nula *zip3,* visto que tanto EVs quanto GXM já foram descritas como capazes de modular processos celulares e células do sistema imune (DE OLIVEIRA, H. C. *et al.*, 2020; ELLERBROEK *et al.*, 2004; FONSECA *et al.*, 2010).

Por fim, os dois sistemas de importação de manganês avaliados nesse trabalho se mostraram importantes para a virulência completa desse fungo em um modelo não-mamífero de infecção. Larvas de *Galleria mellonella* são usadas em ensaios de infecção de microrganismos e avaliação de ação antimicrobiana de drogas *in vivo* (TSAI; LOH; PROFT, 2016). Embora insetos não possuam sistema imune adaptativo, a sua resposta inata a patógenos é extremamente similar a dos vertebrados. Ainda, as larvas toleram temperaturas elevadas como 37°C, mimetizando a temperatura corporal de hospedeiros mamíferos (FIRACATIVE; DUAN; MEYER, 2014). Estudos comparando o modelo clássico murino ao modelo inseto conservaram fenótipos de hiper e hipovirulência entre os modelos (FIRACATIVE; DUAN; MEYER, 2014; MYLONAKIS *et al.*, 2005). Além disso, estratégias de virulência de *Cryptococcus*, como melanização, aumento do diâmetro da cápsula, secreção de vesículas extracelulares, formação de células gigantes e replicação no interior de fagócitos já foram observados no modelo de

infecção inseto (EISENMAN *et al.*, 2014; GARCÍA-RODAS *et al.*, 2011; TREVIJANO-CONTADOR *et al.*, 2015).

Smf1, provavelmente, é importante no contexto de aquisição de manganês em condições escassas do metal, como no sítio de infecção, assim como demonstrado em bactérias. Já Zip3 parece estar relacionado com diversos processos celulares que podem estar influenciando a diminuição na virulência observada, como já discutido anteriormente para o desbalanço redox, alteração de vias de homeostase interna de metal e secreção de determinantes de virulência. A própria redução na taxa de fagocitose dessa linhagem pode estar relacionada a esse fenótipo também, visto que altas taxas de fagocitose são comumente associadas a linhagens hipervirulentas nesse fungo (BOJARCZUK *et al.*, 2016; MANSOUR *et al.*, 2011). Ainda, a inibição química da fagocitose em larvas de *G. mellonella* resultou na diminuição da virulência desse patógeno e um melhor prognóstico da infecção (LIM *et al.*, 2018).

Como o hospedeiro dispõe da habilidade de privar patógenos fúngicos de íons de manganês e a perturbação da homeostase desse metal em patógenos fúngicos afeta diversos processos celulares, incluindo a virulência, é plausível assumir que o hospedeiro utiliza de estratégias semelhantes as utilizadas contra outros patógenos para combater patógenos fúngicos. Dessa forma, nosso trabalho contribui com o ainda raso entendimento sobre o controle homeostático desse metal e sua associação com a patogenicidade de *C. gattii.* Também reforça a ideia de que o hospedeiro utiliza de mecanismos relacionados a imunidade nutricional de manganês para se defender contra patógenos fúngicos.

5. CONCLUSÕES

O presente trabalho demonstrou a importância da correta manutenção da homeostase de manganês e seu impacto no funcionamento celular e na virulência de *Cryptococcus gattii*. Embora preliminares, nossos dados apontam que:

- Zip3 está envolvido em vias de transporte intracelular de metais
- Como transportador predito no Golgi, Zip3 afeta a secreção de diversos determinantes de virulência
- Zip3 está envolvido no correto balanço redox celular
- Smf1 é necessário para aquisição de metais em condições de escassez do mesmo
- Ambos transportadores Smf1 e Zip3 são necessários para completa virulência de *C. gattii* em modelo inseto de infecção

6. PERSPECTIVAS

- Construir linhagem complementada para o gene *SMF1* em *C. gattii*
- Avaliar o balanço oxidativo na linhagem nula *smf1* levando em consideração níveis intracelulares de ROS e sensibilidade a agentes oxidantes
- Avaliar o estado redox de mutantes nulos *smf1* através da expressão de genes relacionados a defesa ao estresse oxidativo
- Avaliar o desfecho da interação entre linhagens nulas *smf1* e macrófagos NRAMP1$^{-/-}$ ou NRAMP1$^{+/+}$ *in vitro*

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8. CURRICULUM VITAE RESUMIDO

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Formação:
Bacharelado em Biomedicina (UFRGS, 2014-2018)

Coleta de sangue (UFRGS, 2015)

Gerenciamento de referências (UFRGS, 2017)

Estágios:
Biologia Celular e Molecular (2016/2 – 2017/2) – Bolsista FAPERGS (UFRGS) – Prof. Dr. Charley Christian Staas – Homeostase de zinco em linhagens de macrófago expostas a *Cryptococcus spp*.

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