UNIVERSIDADE FEDERAL DO RIO GRANDE DO SUL

CENTRO DE BIOTECNOLOGIA

PROGRAMA DE PÓS-GRADUAÇÃO EM BIOLOGIA CELULAR E MOLECULAR

Análise funcional de genes de *Cryptococcus neoformans* envolvidos no processo de interação patógeno-hospedeiro

TESE DE DOUTORADO

Lívia Kmetzsch Rosa e Silva

Porto Alegre, agosto de 2010

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

Lívia Kmetzsch Rosa e Silva

Orientadora: Prof. Dra. Marilene Henning Vainstein

Co-orientador: Prof. Dr. Augusto Schrank

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Sumário

Lista de abreviaturas, símbolos e unidades

°C	graus Celsius
AIDS	*Acquired Immunodeficiency Syndrome*
cAMP	adenosina 3',5' monofosfato cíclico
Ca^{2+}	íon cálcio
CDC	*Centers for Disease Control and Prevention*
cDNA	DNA complementar
CO_2	dióxido de carbono
DNA	ácido desoxirribonucléico
g	força gravitacional
GalXM	galactoxilomanana
GDP	guanosina 5'-difosfato
GXM	glicuronoxilomanana
h	hora
HIV	*Human Immunodeficiency Virus*
kb	kilobase, 1.000 pares de bases
LB	meio de Luria-Bertani
M	molar
MAP	proteína ativada por mitógeno ou *mitogen-activated protein*
min	minuto
ml	mililitros
mM	milimolar
NCR	nitrogen catabolite repression
ng	nanograma
nm	nanômetro
pb ou bp	pares de bases ou base pairs
PCR	reação em cadeia da DNA polimerase
RDA	Análise da Diferença Representacional
RNA	ácido ribonucleico
rpm	rotações por minuto
RT	transcriptase reversa
SAGE	*Serial Analysis of Gene Expression*

T-DNA	DNA de transferência
UDP	uridina 5’-difosfato
UV	radiação ultravioleta
var	variedade
µg	micrograma
µl	microlitro
µM	micromolar
WT	*wild type*
YPD	yeast extract – peptone – dextrose

Lista de Figuras

Resumo

A expressão de determinantes de virulência por um patógeno é amplamente controlada pelo ambiente encontrado no hospedeiro. Neste contexto, a descrição de genes essenciais para o processo de interação patógeno-hospedeiro é fundamental para o melhor entendimento dos mecanismos de virulência utilizados durante a infecção. *Cryptococcus neoformans* é uma levedura encapsulada que causa meningite em pacientes imunocomprometidos. A pandemia de AIDS contribuiu significativamente para o aumento das pesquisas científicas relacionadas à biologia de *C. neoformans*. Estudos funcionais de três genes deste patógeno, *VCX1*, *GAT1*, e *GRASP*, envolvidos, respectivamente, no transporte de cálcio (Ca^{2+}), no metabolismo de nitrogênio e no processo de secreção não convencional são apresentados e discutidos na presente Tese. A habilidade de desevolvimento a 37°C é um fator de virulência essencial de *C. neoformans*, o qual é controlado por uma via de sinalização regulada por Ca^{2+} e calcineurina. O gene *VCX1* de *C. neoformans* codifica um transportador de cálcio vacuolar, requerido para o desenvolvimento intracelular em macrófagos e para a virulência em camundongos. O fator de transcrição Gat1 de *C. neoformans* regula a expressão de genes sensíveis ao processo de repressão catabólica por nitrogênio (NCR). Genes envolvidos na biossíntese de ergosterol, metabolismo de ferro, integridade da parede celular e síntese da cápsula também são regulados por Gat1. Entretanto, este fator de transcrição não é necessário para a sobrevivência durante a interação com macrófagos e para a virulência em modelo de infecção experimental. Uma estratégia de virulência essencial de *C. neoformans* é a secreção de glicuronoxilomanana (GXM), um polissacarídeo capsular com propriedades imuno-modulatórias. Nossos resultados demonstram que GRASP (*golgi reassembly and stacking protein*) está envolvida na secreção de GXM, formação da cápsula e virulência em *C. neoformans*. É importante ressaltar que o transportador de cálcio Vcx1 e o fator de transcrição Gat1 também participam do processo de secreção deste polissacarídeo, o que representa a sua complexidade em *C. neoformans*.

Abstract

The expression of pathogen virulence determinants is highly controlled by the host milieu. In this context, the description of crucial genes for host-pathogen interaction is fundamental to better understand the virulence mechanisms utilized during infection. *Cryptococcus neoformans* is an encapsulated yeast, that causes a life-threatening meningoencephalitis in immunocompromised individuals. The AIDS pandemic has contributed significantly to the increase of scientific research concerning the *C. neoformans* biology. Functional analyses of three genes, *VCX1*, *GAT1*, and *GRASP*, related to calcium (Ca^{2+}) transport, nitrogen metabolism and unconventional secretion in *C. neoformans*, respectively, are presented and discussed in this thesis. The ability to survive and proliferate at the human body temperature is an essential virulence attribute of this pathogen. This trait is controlled in part by the Ca^{2+}-calcineurin pathway, which senses and utilizes cytosolic calcium for signaling. *C. neoformans VCX1* gene encodes a vacuolar calcium exchanger, which is required for intracellular growth in macrophages and for full virulence in mice. The *C. neoformans* transcription factor Gat1 regulates the expression of *Nitrogen Catabolite Repression* (NCR) sensitive genes. Genes involved in ergosterol biosynthesis, iron uptake, cell wall organization and capsule biosynthesis are also Gat1-regulated in *C. neoformans*. However, Gat1 is not required for survival during macrophage infection and for virulence in a mice model of cryptococcosis. One essential virulence strategy of *C. neoformans* is the release of glucuronoxylomannan (GXM), a capsular immune-modulatory polysaccharide. Our results demonstrate that GRASP, a golgi reassembly and stacking protein, is involved in GXM secretion, capsule formation and virulence in *C. neoformans*. Interestingly, the vacuolar calcium exchanger Vcx1 and the transcription factor Gat1 are also involved in GXM secretion, which represents the complexity of this important process in *C. neoformans*.

1. **Introdução**

Estudos relacionados à biologia do patógeno humano *Cryptococcus neoformans* têm assumido papel relevante nos últimos anos. *C. neoformans* é o agente etiológico da meningite criptocócica, infecção fúngica que mais acomete o sistema nervoso central, sendo a terceira complicação neurológica mais frequente em pacientes com a síndrome da imunodeficiência adquirida (AIDS, do inglês, *Acquired Immunodeficiency Syndrome*) (DEL VALLE & PINA-OVIEDO, 2006). Uma recente estimativa global aponta que, por ano, até 12% da população mundial portadora do vírus da imunodeficiência humana (HIV, do inglês *Human Immunodeficiency Virus*) desenvolve esta doença. Em alguns países do continente africano, *C. neoformans* desponta como a causa mais comum de meningite, fato associado a uma maior prevalência de HIV/AIDS na população residente (PARK *et al.*, 2009). Dados disponibilizados pelo *Center for Disease Control and Prevention* (CDC), órgão responsável pelo controle e prevenção de doenças dos Estados Unidos, indicam que a incidência de criptococose por ano varia de 7 a 100 casos por 1.000 habitantes portadores de HIV. No Brasil, *C. neoformans* é o principal causador de micose sistêmica associada à pacientes com AIDS, sendo a criptococose a doença responsável por 51% dos óbitos nestes indivíduos (PRADO *et al.*, 2009).

A descrição de produtos gênicos cruciais para o processo de interação patógeno-hospedeiro é fundamental para um melhor entendimento dos mecanismos de virulência (CASADEVALL & PIROFSKI, 2001). *C. neoformans* tornou-se um modelo para estudos moleculares de mecanismos envolvidos no processo de patogenicidade em fungos. A disponibilidade da sequência

completa do genoma da levedura (LOFTUS *et al.*, 2005), aliada às análises de expressão gênica global e nocaute gênico, tem proporcionado grandes avanços no que diz respeito à identificação de possíveis alvos para o desenvolvimento de novos medicamentos e vacinas (LIU *et al.*, 2008; MA & MAY, 2009).

1.1. Aspectos gerais

C. neoformans foi descrito como patógeno humano em 1894, isolado de uma lesão na tíbia. A recuperação desta levedura a partir de lesões em humanos e em animais, e seu isolamento do ambiente estabeleceram o potencial patogênico e a capacidade de vida livre, respectivamente. O cultivo de *C. neoformans* em condições controladas e a determinação de sua patogenicidade em modelos de infecção experimental propiciaram avanços nos estudos deste patógeno (CASADEVALL & PERFECT, 1998).

C. neoformans e *Cryptococcus gattii* são leveduras pertencentes ao filo *Basidiomycota*, capazes de infectar o sistema nervoso central, os pulmões e a pele do hospedeiro (LIN & HEITMAN, 2006). *C. gattii*, anteriormente classificado como uma variedade de *C. neoformans* (*C. neoformans* var. *gattii*), foi elevado ao nível de espécie, baseado em dados de variabilidade genética e ausência de evidências de recombinação com *C. neoformans* (KWON-CHUNG *et al*., 2002). *C. neoformans* está classificado em duas variedades e três sorotipos (A, D, e o híbrido AD) de acordo com características antigênicas da cápsula polissacarídica, apresentando diferentes propriedades de virulência. *C. neoformans* var. *grubbi* (sorotipo A) (FRANZOT *et al.*, 1999) e *C. neoformans*

var. *neoformans* (sorotipo D) (SORRELL *et al.*, 1996) frequentemente acometem indivíduos imunocomprometidos, tais como portadores de HIV ou pacientes em tratamento com drogas imunossupressoras, apresentando distribuição mundial (CASADEVALL & PERFECT, 1998). É importante ressaltar que linhagens pertencentes ao sorotipo A são responsáveis por 95% das infecções causadas por *C. neoformans* (HULL & HEITMAN, 2002). *C. gattii* (sorotipos B e C) (KWON-CHUNG *et al.*, 2002) tipicamente acomete indivíduos imunocompetentes. Esta espécie é considerada restrita a regiões de clima tropical e subtropical (SORRELL, 2001), porém há registros de um surto de criptococose causada por *C. gattii* em Vancouver, Canadá, região de clima temperado (KIDD *et al.*, 2004). Evidências recentes comprovam que este surto está se expandindo para a região noroeste dos Estados Unidos, e que, predominantemente, o tipo molecular VGII de *C. gattii* é isolado dos pacientes infectados (BYRNES *et al.*, 2010).

A presença ubíqua de *C. neoformans* no ambiente indica que a infecção humana é adquirida de fontes ambientais (CASADEVALL & PERFECT, 1998). Os representantes dos sorotipos A e D de *C. neoformans* geralmente são encontrados no solo e em excretas de aves, principalmente pombos (*Columba livia*), os quais são considerados a maior fonte ambiental desta levedura (CASADEVALL & PERFECT, 1998; CASALI *et al.*, 2003; ABEGG *et al*., 2006; LUGARINI *et al*., 2008). *C. gattii* está predominantemente associado a árvores de eucaliptos (*Eucalyptus* spp.) e madeiras em decomposição (SORRELL, 2001). No Rio Grande do Sul, *C. neoformans* var. *neoformans* foi predominantemente isolada de árvores de eucaliptos (MEDEIROS RIBEIRO *et*

al., 2006). A infecção por *C. neoformans* ocorre por inalação de partículas infecciosas (células leveduriformes) presentes no ambiente (LIN & HEITMAN, 2006). Esporos de *C. neoformans* são também potenciais propágulos infecciosos, visto que há evidências de que tais esporos são patogênicos em modelo de infecção experimental em camundongos e, adicionalmente, são fagocitados por macrófagos alveolares *in vitro* (GILES *et al.*, 2009). *C. neoformans* pode colonizar o trato respiratório do hospedeiro sem causar doença. Há casos de infecção assintomática, com a permanência latente do microrganismo no corpo do indivíduo infectado. Em situações de comprometimento do sistema imune, a forma latente pode ser reativada, permitindo a proliferação do fungo e desenvolvimento de uma infecção sistêmica. Aliado a propensão à disseminação para o sistema nervoso central, podem ocorrer infecções localizadas na pele, olhos, coração, ossos, e trato urinário (LIN & HEITMAN, 2006). O ciclo de infecção de *C. neoformans* e *C. gattii* está evidenciado na Figura 1.

C. neoformans pode ser isolado tanto de pacientes quanto do ambiente na forma leveduriforme. Entretanto, este microrganismo pode sofrer uma transição dimórfica, estabelecendo o desenvolvimento da forma filamentosa. Este processo é controlado por duas rotas de diferenciação distintas: *mating* e frutificação monocariótica. Ambas as vias levam à produção de basidiósporos após o processo de meiose (IDNURM *et al.*, 2005).

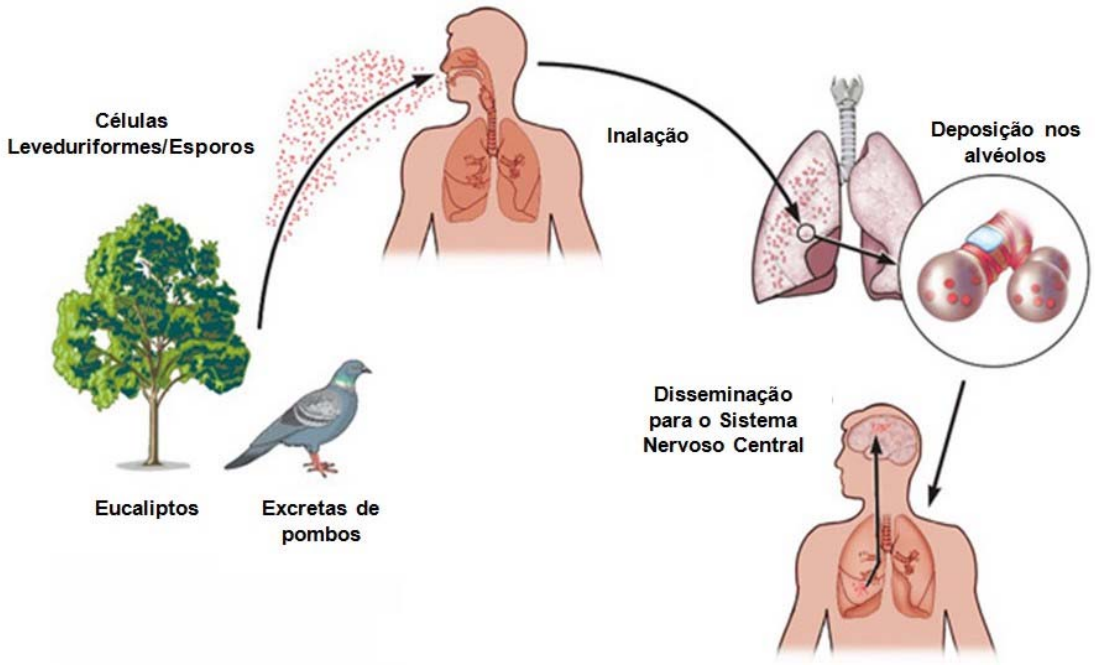

Figura 1. **Ciclo de infecção de *C. neoformans* e *C. gattii*.** No ambiente, *C. neoformans* e *C. gattii* estão, predominantemente, associados à excretas de pombos e árvores de eucaliptos, respectivamente. A infecção em humanos ocorre por inalação de propágulos infecciosos (células leveduriformes ou esporos). Estas células se depositam nos alvéolos pulmonares, podendo ocorrer subsequente disseminação para o Sistema Nervoso Central. Adaptação de imagem disponível em http://www.bmolchem.wisc.edu/labs/hull/research.html.

O ciclo sexual de *C. neoformans* (*mating*) envolve a fusão de células haplóides de *mating types* opostos, α e **a** (KWON-CHUNG, 1976; NIELSEN *et al.*, 2003) (Figura 2). Este ciclo é controlado por mais de 20 genes de *C. neoformans,* organizados no *locus* MAT (LENGELER *et al.*, 2002; IDNURM *et al.*, 2005).

Durante o processo de frutificação monocariótica, células de um mesmo *mating* sofrem diploidização em resposta à limitação de nutrientes. Estudos genotípicos de linhagens de *C. gattii* relacionadas ao surto de criptococose em Vancouver, Canadá (KIDD *et al.*, 2004), evidenciaram ocorrência de

reprodução sexual entre linhagens de *mating type* α (FRASER *et al.*, 2005). Um aspecto interessante desse processo é a ocorrência predominante em linhagens de *mating type* α, podendo, assim, justificar a predominância deste *mating type* em amostras ambientais e clínicas (TSCHARKE *et al.*, 2003; IDNURM *et al.*, 2005).

Existem diferenças significativas na apresentação clínica da doença em relação às infecções causadas por *C. neoformans* e *C. gattii*. A doença decorrente de infecção por *C. gattii* é caracterizada por uma alta incidência de criptococomas no pulmão e no cérebro, que necessitam, frequentemente, de intervenção cirúrgica. Esta infecção apresenta resposta retardada a terapia antifúngica. Já a criptococose causada por *C. neoformans* é associada a lesões pulmonares e cerebrais, as quais se apresentam como infiltrados mais difusos. Esta doença apresenta um maior índice de mortalidade em relação àquela causada por *C. gattii* (MITCHELL & PERFECT, 1995; MA & MAY, 2009).

Atualmente, os medicamentos utilizados para o tratamento da criptococose são os antifúngicos anfotericina B, fluconazol e flucitosina, porém estes possuem efeitos tóxicos para o organismo do paciente. Além disto, o número de linhagens de *C. neoformans* resistentes tem aumentado, tornando-se necessária a descoberta de novos alvos para intervenção terapêutica da doença (ODOM *et al.*, 1997; SANGUINETTI *et al.*, 2006; MA & MAY, 2009). O tratamento de enfermidades causadas por fungos é um desafio, uma vez que existem muitas similaridades entre a maquinaria celular de fungos e a humana, diminuindo-se, desta forma, a disponibilidade de drogas para o tratamento

destas doenças, quando comparadas a doenças causadas por bactérias ou vírus (IDNURM *et al.*, 2005).

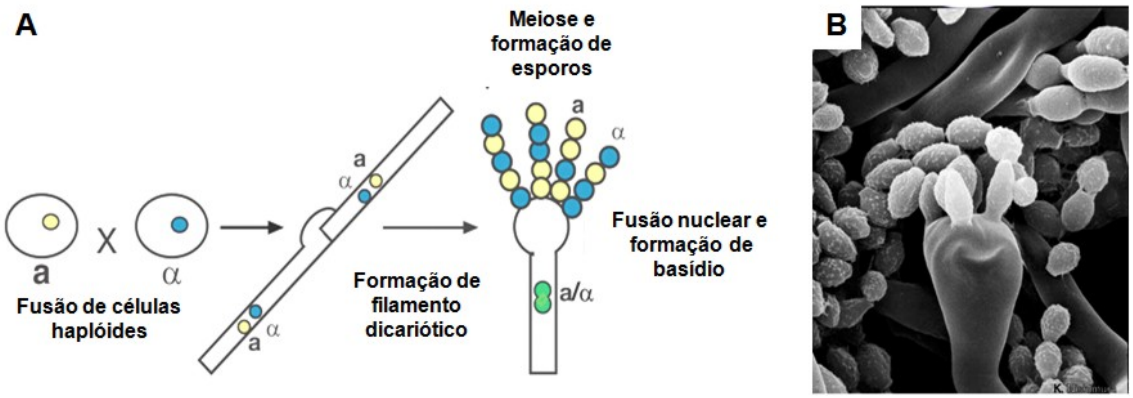

Figura 2. **Ciclo sexual em *C. neoformans*. A.** Durante o ciclo sexual da levedura (*mating*) ocorre fusão de células leveduriformes haplóides de *mating types* opostos, α e a. Formam-se hifas dicarióticas, as quais sofrem um processo meiótico de divisão celular, resultando na produção de basídios contendo basidiósporos. **B.** Imagem gerada por microscopia eletrônica de varredura evidenciando um basídio e inúmeros basidiósporos de *C. neoformans*. Adaptações de imagens disponíveis em http://www.bmolchem.wisc.edu/labs/hull/research.html (A) e http://www.pf.chiba-u.ac.jp/ (B).

O sequenciamento dos genomas de três linhagens de *C. neoformans* (B3501-A, JEC21, H99) e duas linhagens de *C. gattii* (R265, WM276) está completo (LOFTUS *et al.*, 2005; HU *et al.*, 2008). Inicialmente, a sequência nucleotídica de 14 cromossomos, compreendendo um total de 20 Mb de DNA, foi determinada para duas linhagens do sorotipo D de *C. neoformans* var. *neoformans* (B3501-A e JEC21), sendo um passo importante na elucidação do processo de virulência desta levedura patogênica em nível genômico (LOFTUS *et al.*, 2005). A linhagem B3501-A é termotolerante e possui virulência acentuada quando comparada à linhagem JEC21 em modelos de infecção experimental. Aproximadamente 50% da sequência genômica de ambas as

linhagens é idêntica, ao passo que os 50% restantes contêm pequenos polimorfismos. Foram descritos 6.572 genes, os quais contêm uma média de 6,3 éxons de 255 pb e 5,3 íntrons de 67 pb. Eventos de *splicing* alternativo e transcritos antisenso foram identificados, sugerindo a atuação de ambos como mecanismos regulatórios em *C. neoformans* (IDNURM *et al.*, 2005). O sequenciamento completo da linhagem H99 de *C. neoformans* (sorotipo A), assim como da linhagem de *C. gattii* R265 (sorotipo B), está disponível para a comunidade científica na plataforma de sequenciamento de genomas (*Genome Sequencing Platform, Fungal Genome Initiative*) do *Broad Institute* (http://www.broadinstitute.org/). A disponibilidade do sequenciamento completo dos genomas de *C. neoformans* e *C. gattii* auxilia na elucidação de diferenças nas estratégias de virulência entre estas leveduras e outros fungos patogênicos, bem como na identificação e caracterização de genes envolvidos na patogenicidade (LOFTUS *et al.*, 2005; MA & MAY, 2009).

1.2. *C. neoformans* e Virulência

A virulência de determinado microrganismo varia de acordo com a suscetibilidade do hospedeiro ao patógeno que está causando infecção. Neste contexto, o controle da expressão de determinantes de virulência pelo patógeno é predominantemente dependente da interação patógeno-hospedeiro (CASADEVALL & PIROFSKI, 2001). *C. neoformans* possui alguns fatores de virulência bem definidos e caracterizados, os quais incluem: (i) elaboração de uma cápsula polissacarídica; (ii) síntese de melanina; e, (iii) habilidade de desenvolvimento a 37°C (CASADEVALL & PERFECT, 1998; MCCLELLAND *et al.*, 2005). Estes fatores, assim como as principais vias de sinalização e

aspectos relacionados à interação patógeno-hospedeiro serão discutidos, a seguir, em detalhes.

1.2.1. Cápsula polissacarídica

A cápsula polissacarídica foi o primeiro fator de virulência de *C. neoformans* a ser associado à patogênese, e é considerada um dos principais determinantes de virulência (CASADEVALL & PERFECT, 1998) (Figura 3). Sua composição é de aproximadamente 90% de glicuronoxilomanana (GXM), 5% de galactoxilomanana (GalXM) e, o restante, corresponde a manoproteínas. O principal polissacarídeo da cápsula de *C. neoformans*, GXM, possui função relevante durante a interação patógeno-hospedeiro. Acredita-se que pelo menos 12 glicosiltransferases são necessárias para a síntese deste polissacarídeo (DOERING, 2000; KLUTTS *et al.*, 2006). Estas enzimas atuam na formação das fibras de GXM, transferindo monossacarídeos para uma molécula aceptora. As unidades básicas doadoras para a formação das fibras são: UDP-ácido glicurônico, UDP-xilose e GDP-manose. A síntese de GXM é realizada dentro da célula, mais especificamente, no complexo de Golgi (YONEDA & DOERING, 2006). O transporte deste polissacarídeo para a superfície da célula é mediado por secreção de vesículas que contêm GXM e que, de alguma forma, ultrapassam a parede celular, liberando seu conteúdo no meio extracelular (RODRIGUES *et al.*, 2007). Sugere-se que a montagem da cápsula é resultante de um processo de auto-agregação de fibras de GXM acumuladas no meio extracelular, mediada pela ação de cátions divalentes (NIMRICHTER *et al.*, 2007). É importante ressaltar que estas vesículas, correlacionadas a estruturas do tipo exossomo, transportam para o meio

extracelular, além de GXM, uma série de proteínas relacionadas à virulência, tais como lacase, urease e fosfolipase B (RODRIGUES *et al.*, 2008). Além disto, há alguns estudos que comprovam a secreção de tais vesículas durante infecção experimental e a modulação da função de macrófagos pelas mesmas (RODRIGUES *et al.*, 2008; OLIVEIRA *et al.,* 2010). A exposição de *C. neoformans* à brefeldina A, composto que afeta a formação de vesículas relacionadas ao complexo de Golgi, resulta em uma extrema inibição da montagem da cápsula polissacarídica (HU *et al.*, 2007).

Em estudos de infecção experimental em camundongos, a composição antigênica da cápsula *in vivo* é distinta em diferentes órgãos infectados, sugerindo uma evolução da estrutura capsular durante a infecção (CHARLIER *et al.*, 2005). A acetilação também é um importante fator responsável pelas propriedades imunogênicas e de virulência da cápsula (KOZEL *et al.*, 2003). Durante a infecção, há um aumento significante de seu tamanho devido à presença de estímulos, tais como altos níveis de dióxido de carbono (CO_2) e limitação de ferro. Há evidências de que o tamanho da cápsula varia de acordo com o órgão infectado, sendo que células isoladas do pulmão são as que apresentam o maior tamanho de cápsula observado. Além disto, as propriedades imunogênicas da cápsula podem se alterar durante o curso da infecção, representando uma adaptação para sobrevivência no hospedeiro (ZARAGOZA *et al.*, 2009). Sabe-se que o desenvolvimento da cápsula ocorre por crescimento apical, pela adição de fibras de polissacarídeos secretadas no meio extracelular, e que durante o brotamento, forma de reprodução assexuada da levedura, há a formação de rearranjos na estrutura capsular

(ZARAGOZA *et al.*, 2006; FRASES *et al.*, 2009). O ancoramento da cápsula à parede celular de *C. neoformans* requer a ação de α-1,3 glicana, quitina e N-acetilglicosamina (RODRIGUES *et al.*, 2008; FONSECA *et al.*, 2009).

A cápsula também está presente em isolados ambientais, envolvendo a parede celular da célula fúngica, com a provável função de proteção contra a desidratação. Esta estrutura confere efeitos benéficos a *C. neoformans* durante a infecção, atuando como uma barreira física, que interfere no processo de fagocitose por macrófagos e dificulta a ação do sistema imune do hospedeiro (ZARAGOZA *et al.*, 2009). Componentes da cápsula inibem a produção de citocinas pró-inflamatórias, inativam componentes do sistema complemento e reduzem a migração de leucócitos ao sítio de infecção (BOSE *et al.*, 2003). Análises das propriedades imunossupressivas de GXM revelaram que este componente é capaz de induzir apoptose em células T do sistema imune do hospedeiro (MONARI *et al.*, 2006). Existem inúmeras evidências que apontam a modulação da função de fagócitos mediada por componentes da cápsula polissacarídica (VECCHIARELLI *et al.*, 2003; YAUCH *et al.*, 2006; VILLENA *et al.*, 2008; ZARAGOZA *et al.*, 2009).

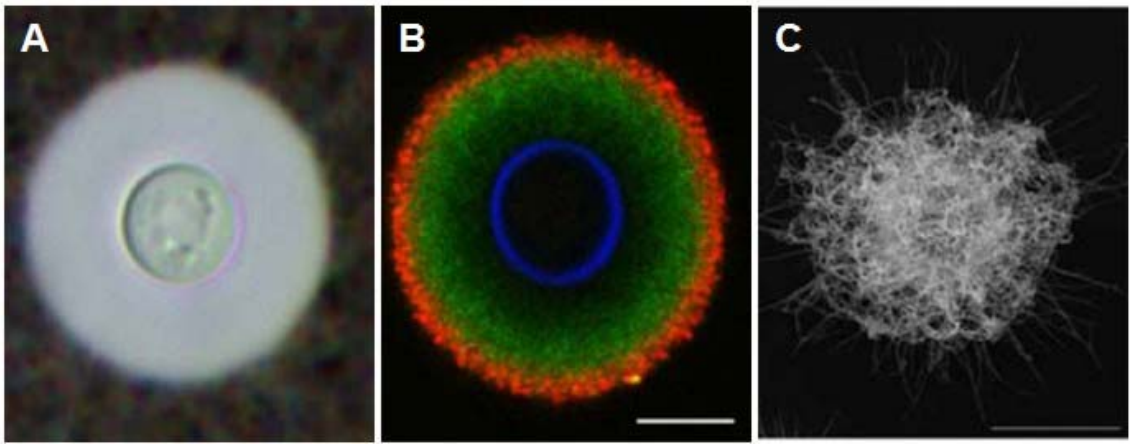

Figura 3. **Cápsula polissacarídica de *C. neoformans***. **A.** Imagem gerada por microscopia óptica de uma célula de *C. neoformans* em presença de tinta da China. **B.** Imagem resultante de microscopia de fluorescência de uma célula de *C. neoformans* marcada com anticorpos específicos para a cápsula (verde e vermelho). A parede celular está evidenciada em azul, por marcação com *Calcofluor White*. **C.** Imagem gerada por microscopia eletrônica de varredura de célula de *C. neoformans*, evidenciando as fibras que compõem a cápsula polissacarídica. Adaptações de imagens disponíveis em ZARAGOZA *et al.*, 2009.

A estrutura polissacarídica da cápsula é complexa, sendo que sua síntese requer uma série de produtos gênicos (DOERING, 2000). Análises por complementação de mutantes de linhagens de *C. neoformans* desprovidas de cápsula possibilitaram a identificação de quatro genes necessários para a sua produção. Os genes *CAP* (*CAP59*, *CAP60*, *CAP10*, *CAP64*), quando analisados por disrupção gênica, mostraram-se essenciais para a síntese de cápsula e para a virulência, enfatizando a importância da cápsula durante o processo de interação patógeno-hospedeiro (CHANG & KWON-CHUNG, 1994; CHANG *et al.*, 1996; CHANG & KWON-CHUNG, 1998; 1999). Um segundo grupo de genes também parece exercer um papel fundamental nesta rota. Os genes *CAS* estão envolvidos no processo de acetilação de GXM e são associados à virulência (MOYRAND *et al.*, 2004). Além disto, genes

relacionados aos mais diversos processos biológicos, como metabolismo de ferro (*CIR1*), vias de transdução de sinal (*PKA1*, *GPR4*, *ACA1*) e metabolismo de carboidratos (*NRG1*) também regulam a síntese de cápsula em *C. neoformans* (D'SOUZA & HEITMAN, 2001; BAHN *et al.*, 2004; LIAN *et al.*, 2005; CRAMER *et al.*, 2006; XUE *et al.*, 2006).

1.2.2. Melanina

A produção de melanina contribui para a virulência de *C. neoformans* protegendo a levedura contra os mecanismos do sistema de defesa do hospedeiro. Melaninas são pigmentos hidrofóbicos carregados negativamente, formados pela oxidação de compostos fenólicos e/ou indólicos. O fungo capta precursores dopaminérgicos do ambiente extracelular e, por ação da enzima lacase associada à membrana, sintetiza melanina, com sua consequente deposição entre a membrana e a parede celular (CASADEVALL & PERFECT, 1998; ZHU *et al.*, 2001) (Figura 4). No ambiente, a melanização é um mecanismo de proteção contra predadores como, por exemplo, amebas (STEENBERGEN *et al.*, 2001), ou contra radiação ultravioleta (UV) (CASADEVALL & PERFECT, 1998). Durante a infecção, a produção de melanina inibe a resposta imune do hospedeiro, protege as células fúngicas contra antioxidantes, e contra a ação de macrófagos (ZHU & WILLIAMSON, 2004). Células de *C. neoformans* recuperadas de tecido cerebral humano são melanizadas, ressaltando a importância deste fator de virulência durante a infecção (NOSANCHUK *et al.*, 2000). A síntese de melanina em *C. neoformans* utiliza substratos exógenos, tais como dopamina, epinefrina, e norepinefrina. O tropismo da levedura pelo sistema nervoso central pode-se justificar pela

presença destes compostos em altas concentrações. Áreas do cérebro ricas nestes neurotransmissores são frequentemente colonizadas durante a infecção (CASADEVALL & PERFECT, 1998).

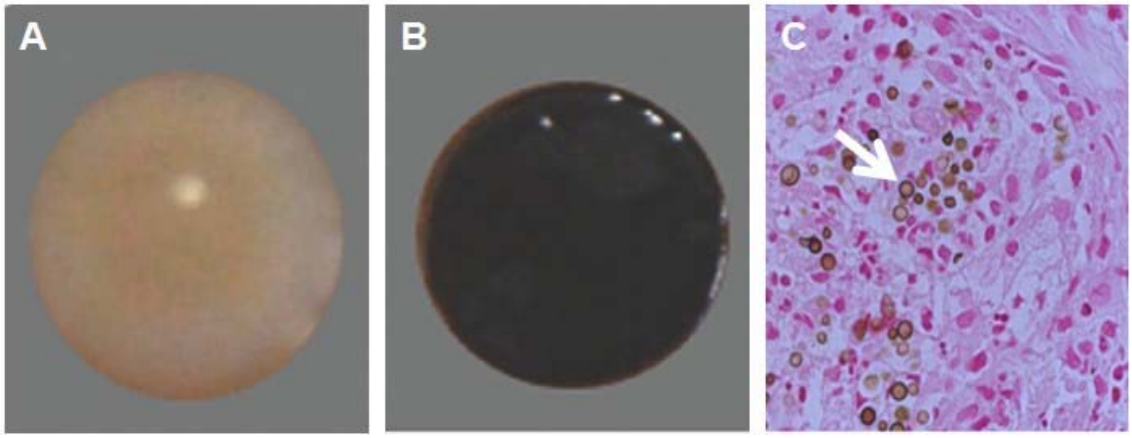

Figura 4. **Produção de melanina por *C. neoformans***. **A**. Colônia de células de *C. neoformans* não melanizadas. **B**. Colônia de células de *C. neoformans* melanizadas. **C**. Imagem gerada por microscopia óptica de tecido prostático humano infectado por *C. neoformans*. Coloração *Fontana-Masson* evidencia a presença de células de *C. neoformans* melanizadas neste tecido, como indicado pela seta. Adaptações de imagens disponíveis em MA & MAY, 2009 (A e B) e WADA *et al*., 2008 (C) (WADA *et al.*, 2008).

A enzima lacase, responsável pela síntese de melanina em *C. neoformans,* é codificada pelo gene *LAC1,* e exerce acentuado impacto na virulência deste microrganismo. Linhagens mutantes para este gene (*lac1*Δ) foram analisadas em modelos de infecção experimental, e mostraram-se menos virulentas quando comparadas a linhagens selvagens (ZHU *et al.,* 2001). Uma segunda lacase, codificada pelo gene *LAC2*, foi descrita em *C. neoformans*. Sabe-se que esta enzima também participa da biossíntese de melanina, porém seu nível de transcrição basal é significativamente menor em relação à transcrição de *LAC1* (ZHU & WILLIAMSON, 2004). Vias de

sinalização ativadas por adenosina 3´,5´ monofosfato cíclico (cAMP) controlam a produção de melanina, bem como a síntese da cápsula polissacarídica. Além disto, genes envolvidos em acidificação vesicular (*VPH1*), homeostase de cobre (*CCC2*, *ATX1*), integridade da parede celular (*CHS3*) e remodelamento de cromatina (*SNF5*) (ERICKSON *et al.*, 2001; WALTON *et al.*, 2005) também estão relacionados à produção deste pigmento.

1.2.3. Habilidade de desenvolvimento a 37°C

Microrganismos patogênicos devem estar aptos a sobreviver na temperatura fisiológica do hospedeiro para que ocorra a proliferação e manutenção da infecção. A habilidade de desenvolvimento a 37ºC é uma característica fundamental de fungos patogênicos humanos. A tolerância a temperaturas elevadas pode variar entre linhagens e variedades de *C. neoformans*. Linhagens de *C. neoformans* var. *grubii* (sorotipo A) geralmente são mais tolerantes, quando comparadas a linhagens de *C. neoformans* var. *neoformans* (sorotipo D) (MARTINEZ *et al.*, 2001).

Estudos moleculares identificaram uma variedade de genes associados ao desenvolvimento a 37ºC em *C. neoformans* (PERFECT, 2005; MA & MAY, 2009). O gene *CNA1* codifica a proteína calcineurina, uma fosfatase serina/treonina específica, necessária para resposta ao estresse e integridade celular em leveduras (ODOM *et al.*, 1997). A calcineurina é formada por um heterodímero composto por uma subunidade catalítica A (*CNA1*), e uma subunidade regulatória B (*CNB1*) (FOX *et al.*, 2001). A associação destas duas subunidades é necessária, porém não essencial, para obtenção da calcineurina

funcional. A ativação ocorre quando complexos Ca^{+2}/calmodulina ligam-se a um domínio localizado na região regulatória C-terminal da subunidade A, desencadeando mudanças conformacionais e exposição do sítio ativo da enzima (KRAUS *et al.*, 2005). Mutantes nulos para calcineurina não são viáveis a 37ºC, e não são patogênicos quando analisados por infecção em modelo experimental (ODOM *et al.*, 1997). Com o intuito de caracterizar componentes de rotas de sinalização reguladas por calcineurina, o gene *CTS1* (*calcineurin temperature supressor*) foi caracterizado. A disrupção deste gene por recombinação homóloga resultou em sensibilidade à temperatura, defeito da separação celular e posicionamento do septo, inibição do desenvolvimento de hifas, e virulência atenuada da linhagem mutante de *C. neoformans* (FOX *et al.*, 2003).

Diversos genes regulados pela temperatura já foram identificados em *C. neoformans*, porém nem todos são essenciais para o desenvolvimento a 37°C (MA & MAY, 2009). Uma análise por hibridização em microarranjos identificou 49 genes que possuem expressão induzida a 37°C, entre eles o fator de transcrição *MGA2*, envolvido na regulação da síntese de ácidos graxos. A expressão de genes envolvidos em processos de remodelamento de membrana parece ser essencial para a adaptação a temperaturas elevadas (KRAUS *et al.*, 2004). De forma similar, um recente estudo utilizando a metodologia de Análise da Diferença Representacional (RDA) descreveu 29 genes regulados por temperatura em *C. neoformans*, apresentando algumas sobreposições em relação aos genes identificados por microarranjo (ROSA E SILVA *et al.*, 2008). Estes genes estão envolvidos, principalmente, em resposta

ao estresse, integridade de membrana e parede celular, e metabolismo celular básico (ROSA E SILVA *et al.*, 2008).

As defesas antioxidantes de *C. neoformans* exercem forte influência na habilidade de desenvolvimento a 37ºC. O produto do gene *SOD2*, o qual codifica uma superóxido dismutase, atua como uma proteção contra os mecanismos de defesa do hospedeiro dependentes de oxigênio (GILES *et al.*, 2005). Análises do proteoma de cultivo de *C. neoformans* a 37ºC identificaram uma tiol peroxidase (TSA1), envolvida em resistência a óxido nítrico e peróxido de hidrogênio. Peroxidases atuam na defesa contra danos oxidativos, e mutantes *tsa1*Δ são significativamente menos virulentos (MISSALL *et al.*, 2004). Macrófagos, células do sistema imune do hospedeiro, produzem óxido nítrico em resposta à infecção por *C. neoformans* (GROSS *et al.*, 1999), e genes envolvidos na resposta a este estresse são essenciais para a manutenção da infecção (MISSALL *et al.*, 2006).

Genes relacionados à síntese de aminoácidos são necessários para a sobrevivência de *C. neoformans* a 37ºC. O gene *SPE3-LYS9* (espermidina sintase-sacaropina desidrogenase) está envolvido na rota sintética de lisina (KINGSBURY *et al.*, 2004a). O gene *ILV2* (acetolactato sintase) atua em rotas de síntese de isoleucina e valina, e mutantes *ivl2*Δ perdem a viabilidade a 37ºC, e são avirulentos em modelos de infecção experimental (KINGSBURY *et al.*, 2004b). Além disto, genes envolvidos na síntese de treonina (*HOM3*, *THR1*) são também requeridos para desenvolvimento na temperatura do hospedeiro humano (KINGSBURY & MCCUSKER, 2008).

1.2.4. Vias de sinalização em *C. neoformans*

Fungos patogênicos utilizam sistemas complexos de sinalização para adaptação a distintos ambientes, os quais modulam desenvolvimento, diferenciação, resposta a estresse e virulência (KOZUBOWSKI *et al.*, 2009; MA & MAY, 2009). Vias de sinalização mediadas por proteína quinase A (PKA) dependente de cAMP, MAP quinase (MAPK), e Ca^{2+}/calcineurina são bem caracterizadas em *C. neoformans* (KOZUBOWSKI *et al.*, 2009).

Em *C. neoformans*, a sinalização mediada por cAMP é desencadeada por estímulos ambientais, principalmente nutricionais, levando a ativação da proteína G Gpa1 (ALSPAUGH *et al.*, 1997). Gpa1 ativa a enzima adenilatociclase (Cac1), resultando em geração de cAMP e subsequente ativação de PKA (PUKKILA-WORLEY & ALSPAUGH, 2004). Importantes processos celulares são regulados por esta via, os quais incluem a produção de cápsula, síntese de melanina e *mating* (MA & MAY, 2009). As vias de sinalização mediadas por MAPK envolvem cascatas de fosforilação que, em *C. neoformans*, regulam resposta a estresse (Hog1-MAPK), diferenciação (Gpb1), integridade da parede celular e desenvolvimento a 37°C (Mpk1) (KOZUBOWSKI *et al.*, 2009).

A via de sinalização mediada por calcineurina em *C. neoformans* controla o desenvolvimento a 37°C, o processo de *mating*, a frutificação monocariótica, a resposta a estresse e a virulência (ODOM *et al.*, 1997; CRUZ *et al.*, 2001; FOX *et al.*, 2001; KOZUBOWSKI *et al.*, 2009) (Figura 5). A presença de Ca^{2+} é fundamental para a ativação desta via de sinalização. Ao

entrar na célula, pela ação de canais de cálcio localizados na membrana (Cch1) (LIU *et al.*, 2006), este mensageiro secundário liga-se a proteína calmodulina (Cam1) (KRAUS *et al.*, 2005), a qual ativa a fosfatase calcineurina. Subsequentemente, a formação deste complexo leva à ativação da expressão de genes necessários para responder a estímulos ambientais e manter a homeostase de cálcio na célula (STIE & FOX, 2008). O controle do processo de *mating* por esta via também é dependente da proteína calcipressina (Cbp1) (FOX & HEITMAN, 2005). Na Figura 5 está resumida a via de sinalização mediada por Ca^{2+}/calcineurina em *C. neoformans,* evidenciando também seus inibidores (Ciclosporina A e FK506).

1.2.5. Interação patógeno-hospedeiro

A virulência dos microrganismos está diretamente relacionada com as interações que ocorrem entre patógenos e hospedeiros durante a infecção. O dano causado ao hospedeiro é resultante tanto da ação de componentes do patógeno quanto da resposta imune gerada pelo hospedeiro (CASADEVALL & PIROFSKI, 2003). Neste contexto, as interações de *C. neoformans* com células fagocíticas do sistema imune inato (neutrófilos, células dendríticas e macrófagos) têm sido amplamente estudadas (FELDMESSER *et al.*, 2000; MAMBULA *et al.*, 2000; TUCKER & CASADEVALL, 2002; MEDNICK *et al.*, 2003; KELLY *et al.*, 2005; WOZNIAK *et al.*, 2006; ALVAREZ & CASADEVALL, 2007; MA *et al.*, 2007). O processo de fagocitose é desencadeado pelo reconhecimento direto de *C. neoformans* no sítio de infecção ou por reconhecimento mediado por anticorpos e componentes do sistema complemento (MITCHELL & PERFECT, 1995).

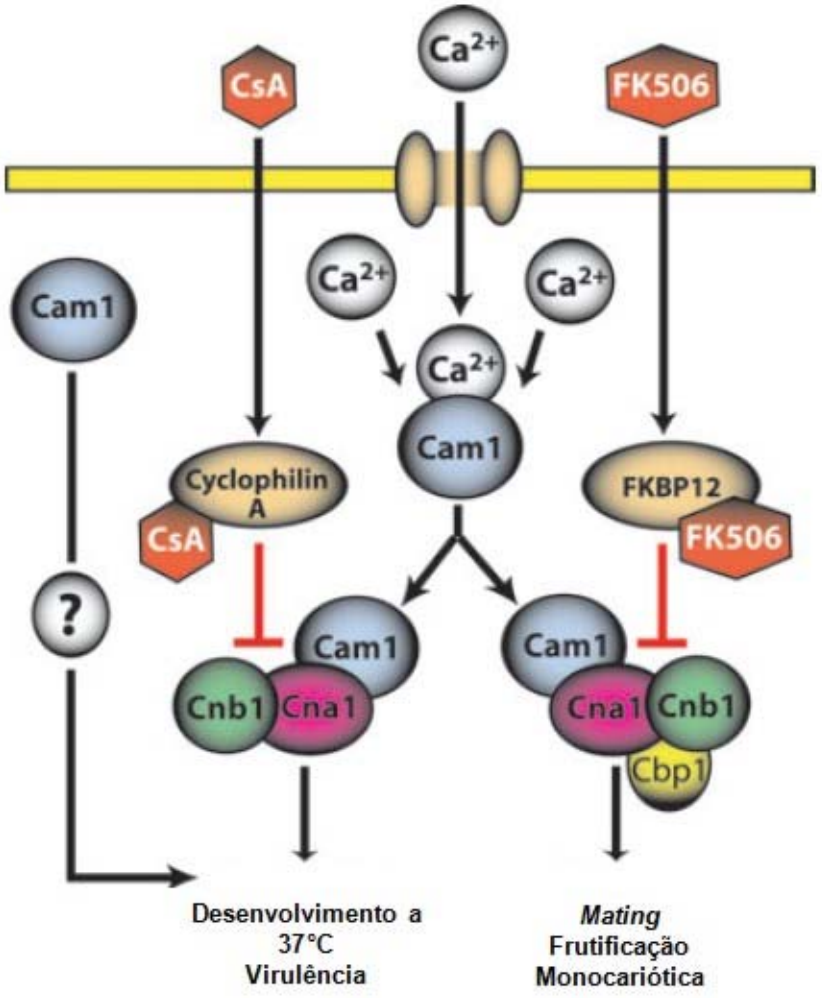

Figura 5. **Via de sinalização mediada por Ca^{2+}/Calcineurina em *C. neoformans***. A entrada de cálcio na célula ocorre pela ação de transportadores presentes na membrana plasmática. A proteína calmodulina (Cam1) atua como um sensor de cálcio citosólico. O complexo Ca^{2+}/Cam1 ativa a proteína calcineurina (representada pelas subunidades Cna1 e Cnb1), resultando na ativação de fatores que regulam o desenvolvimento a 37°C e virulência em *C. neoformans,* os quais podem ser também regulados independentemente por Cam1. Os processos de *mating* e frutificação monocariótica são regulados pelo complexo formado por Ca^{2+}/Cam1, calcineurina (Cna1 e Cnb1) e calcipressina (Cbp1). Os inibidores destas vias de sinalização são as drogas imunossupressoras ciclosporina A (CsA) e FK506, as quais se ligam às proteínas ciclofilina A e FKBP12, respectivamente. Adaptação da imagem disponível em KOZUBOWSKI *et al.* (2009).

Durante a infecção, neutrófilos entram em contato com *C. neoformans* no ambiente pulmonar. Estudos *in vitro* e *in vivo* comprovaram que estas células fagocitam *C. neoformans* eficientemente, porém somente em estágio precoce de infecção (FELDMESSER *et al.*, 2000; MAMBULA *et al.*, 2000). Ensaios de infecção experimental de *C. neoformans* em camundongos com condições de depleção de neutrófilos revelaram que os níveis de IL-4/IL-10 (citocinas Th2) e IL-12/TNFα (citocinas Th1) são significativamente maiores nestes animais em relação aos camundongos controle, sugerindo que a depleção de neutrófilos é protetora durante infecção pulmonar por *C. neoformans*. Além disto, foi observado um aumento da sobrevivência destes camundongos em relação ao controle, fato que pode estar associado à morte mais eficiente do patógeno, desencadeada pela ação de IL-12/TNFα. Além disto, pode haver reduzido dano ao hospedeiro mediado por IL-4/IL-10 (MEDNICK *et al.*, 2003). Estudos recentes mostram que células dendríticas também são capazes de fagocitar células de *C. neoformans* em condições *in vivo* e *in vitro* (KELLY *et al.*, 2005; WOZNIAK *et al.*, 2006). Durante a infecção por *C. neoformans*, estas células atuam na apresentação de antígenos às células T, com o intuito de induzir uma resposta imune adaptativa. Há evidências de que tal indução é mais pronunciada em relação àquela desencadeada por macrófagos alveolares ou monócitos (SYME *et al.*, 2002; MANSOUR *et al.*, 2006).

Estudos relacionados às múltiplas interações de *C. neoformans* com macrófagos apontam que este microrganismo é um patógeno intracelular facultativo (FELDMESSER *et al.*, 2001; VOELZ *et al.*, 2009; VOELZ & MAY,

2010). *C. neoformans* desenvolveu mecanismos peculiares de manipulação dos macrófagos durante a infecção (Figura 6). Sabe-se que, após a fagocitose, pode ocorrer replicação das células de *C. neoformans* seguida de lise dos macrófagos para liberação de células fúngicas viáveis, ou apenas um processo de extrusão, sem ocasionar a morte das células de defesa com subsequente resposta inflamatória (TUCKER & CASADEVALL, 2002; ALVAREZ & CASADEVALL, 2006; MA *et al.*, 2006). *C. neoformans* desenvolveu uma estratégia que envolve a liberação de vesículas contendo polissacarídeo (GXM) no citoplasma do macrófago que, seguida de replicação intracelular, leva a formação de grandes fagossomos. A presença de GXM intracelular altera a função dos macrófagos em múltiplos níveis, evidenciando a importância deste polissacarídeo capsular durante a interação patógeno-hospedeiro (TUCKER & CASADEVALL, 2002). Além disto, existem evidências de que células de *C. neoformans* são disseminadas de um macrófago infectado para um macrófago não infectado, por um processo de transferência lateral (ALVAREZ & CASADEVALL, 2007; MA *et al.*, 2007). Os mesmos mecanismos foram observados durante interação de *C. neoformans* com monócitos humanos (ALVAREZ *et al.*, 2009). Em conjunto, estes fatos comprovam que *C. neoformans* é capaz de modular a função de macrófagos a seu favor durante o estabelecimento e proliferação da infecção (VOELZ & MAY, 2010; MA & MAY, 2009).

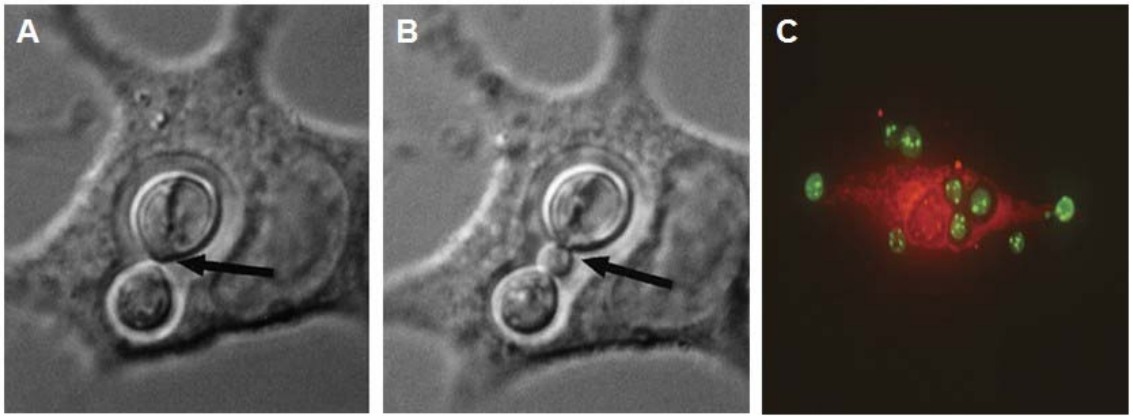

Figura 6. **Interação de *C. neoformans* com o sistema imune do hospedeiro**. **A** e **B**. Células de *C. neoformans* fagocitadas por macrófagos, linhagem celular J774. O evento de replicação intracelular está evidenciado pelas setas. **C**. Imagem gerada por microscopia de fluorescência de células de *C. neoformans* (verde) aderidas e fagocitadas por monócito humano (vermelho). Adaptações de imagens disponíveis em TUCKER & CASADEVALL (2002) (A e B), e NICOLA *et al*. (2008) (C).

A meningite criptocócica é resultante do processo de disseminação das células de *C. neoformans* por via hematogênica dos pulmões para o cérebro humano. Para tanto, é necessário que as células fúngicas atravessem a barreira hematoencefálica. Há dois mecanismos propostos para este processo: invasão direta por transcitose ou transporte mediado por macrófagos (cavalo de Tróia - *Trojan horse mechanism*) (CHANG *et al.*, 2004; CHARLIER *et al.*, 2009; CASADEVALL, 2010). Um estudo recente, utilizando microscopia intravital, sustenta o mecanismo de invasão direta, no qual células de *C. neoformans* atravessam diretamente as células endoteliais após um evento de micro-embolia, gerado pela parada das células fúngicas em pequenos vasos sanguíneos cerebrais. Este processo é dependente de urease, requer viabilidade celular e alterações morfológicas importantes (SHI *et al.,* 2010). Entretanto, também existem evidências que comprovam a ocorrência do

mecanismo de cavalo de Tróia, no qual macrófagos infectados com células fúngicas atravessam do vaso sanguíneo para o parênquima cerebral, indicando que pelo menos duas estratégias funcionais distintas podem ser utilizadas por *C. neoformans* para alcançar o sistema nervoso central (CASADEVALL, 2010).

1.3. Estratégias para identificação e análise funcional de genes envolvidos na virulência de *C. neoformans* e *C. gattii*

C. neoformans e *C. gattii* são excelentes modelos para avaliação da função de genes envolvidos no processo de interação patógeno-hospedeiro. Estes microrganismos geralmente desenvolvem-se como leveduras haplóides, podendo ser facilmente transformados, o que facilita a disrupção gênica por recombinação homóloga (PERFECT, 2005). Sistemas de transformação mediados por eletroporação (EDMAN & KWON-CHUNG, 1990), bombardeamento (TOFFALETTI *et al.*, 1993) ou por *Agrobacterium tumefaciens* (MCCLELLAND *et al.*, 2005), e a utilização de marcas de seleção dominante (MCDADE & COX, 2001) ou complementação auxotrófica (EDMAN & KWON-CHUNG, 1990) para seleção dos transformantes estão bem estabelecidos. A eficiência de recombinação homóloga varia de acordo com o método de transformação e a linhagem que será transformada (PERFECT, 2005). Estratégias envolvendo interferência de RNA (LIU *et al.*, 2002) e repressão antisenso (GORLACH *et al.*, 2002) também já foram aplicadas em *C. neoformans* com o intuito de identificar genes essenciais. Além disto, modelos de infecção experimental para estudos de virulência foram estabelecidos em

camundongos, ratos, coelhos, cultura de células de macrófagos *in vitro*, e em hospedeiros ambientais, incluindo as amebas *Acanthamoeba castellanii* e *Dictyostelium discoideum*, o verme *Caenorhabditis elegans*, a mosca *Drosophila melanogaster,* e larvas do inseto *Galleria mellonella* (IDNURM *et al.*, 2004; LONDON *et al.*, 2006; KROCKENBERGER *et al.*, 2010).

Análises por mutagênese insercional e deleção gênica têm sido adaptadas e desenvolvidas como ferramentas eficientes para investigação da função de genes em fungos (WELD *et al.*, 2006). Experimentos de mutagênese insercional mediada por *A. tumefaciens* permitem a integração aleatória da região de T-DNA no genoma de *C. neoformans* e consequente identificação de novos componentes relacionados a fatores de virulência da levedura (IDNURM *et al.*, 2004). Esta estratégia tem sido utilizada com frequência em *C. neoformans* para a identificação e estudo de genes envolvidos em melanização (WALTON *et al.*, 2005), diferenciação celular (YEH *et al.*, 2009; LIN *et al.,* 2010) e virulência (IDNURM *et al.*, 2009).

Estudos de função gênica baseados em deleção, associados à disponibilidade da sequência completa do genoma de *C. neoformans*, têm permitido a descrição de inúmeros genes envolvidos na virulência e expressos durante a interação patógeno-hospedeiro (IDNURM *et al.*, 2005; LIU *et al.*, 2008; MA & MAY, 2009). Recentemente, Liu *et al*. realizaram uma análise sistemática da função de 1.201 genes de *C. neoformans* por nocaute gênico. Este estudo permitiu a identificação de 164 mutantes com virulência atenuada em modelo de infecção pulmonar em camundongos. Fenótipos relacionados aos principais fatores de virulência de *C. neoformans* também foram avaliados,

levando a identificação de 33 novos genes necessários para a melanização e cinco novos genes necessários para síntese da cápsula polissacarídica (LIU *et al.*, 2008).

A deleção de múltiplos genes em uma mesma linhagem de *C. neoformans* representa uma importante estratégia para o estudo da função de produtos gênicos com funções relacionadas ou de produtos de genes parálogos. O nocaute múltiplo de quatro genes que codificam distintas quitinases (*CHI2*, *CHI21*, *CHI22*, *CHI4*) foi realizado, o que possibilitou a avaliação do papel destas enzimas em processos de desenvolvimento e ciclo sexual de *C. neoformans* (BAKER *et al.*, 2009). Além disto, um mecanismo de reciclagem da marca de seleção foi descrito em *C. neoformans* para este intuito, baseado no sistema Cre-loxP do bacteriófago P1 (PATEL *et al.,* 2010).

Avaliações do perfil de transcrição global de *C. neoformans* em condições controladas são úteis para a identificação de produtos gênicos atuantes em processos de virulência. Análise seriada da expressão gênica (SAGE) foi utilizada para identificar genes de *C. neoformans* transcritos preferencialmente em condições de temperatura elevada (STEEN *et al.*, 2002), limitação de ferro (LIAN *et al.*, 2005) e infecção em modelo experimental (STEEN *et al.*, 2003; HU *et al.*, 2008). Da mesma forma, estudos para o delineamento do padrão de transcrição global de *C. neoformans* em condições de diferenciação celular (LIN *et al.,* 2010), hipóxia (CHANG *et al.*, 2007; LEE *et al.*, 2007) e desenvolvimento a 37ºC (KRAUS *et al.*, 2004) foram realizados utilizando a estratégia de hibridização em microarranjos de DNA. Recentemente, esta mesma metodologia foi empregada por Ma *et al*. para

descrever transcritos diferencialmente expressos por 24 linhagens de *C. gattii* provenientes do surto de criptococose em Vancouver, Canadá. Genes codificados pelo genoma mitocondrial ou associados com atividades da mitocôndria apresentaram aumento de expressão nestas linhagens em relação à linhagem controle. Este fato parece estar associado ao aumento dos níveis de replicação intracelular em macrófagos, e ao fenótipo de hiper virulência destes isolados (MA *et al.*, 2009).

Distintas estratégias, as quais têm sido aplicadas com o intuito de identificar genes relevantes no processo de interação patógeno-hospedeiro, são essenciais para a descrição de fatores indispensáveis para a virulência de *C. neoformans*. A relevância clínica destas descobertas, associada aos avanços na área de genômica funcional, tornaram *C. neoformans* um organismo modelo para estudos de patogenicidade em fungos de importância médica (MA & MAY, 2009). Pela presente Tese estão descritas a identificação e a caracterização funcional de três genes, *VCX1*, *GAT1* e *GRASP*, envolvidos em distintos processos biológicos em *C. neoformans* e potencialmente relacionados ao processo de interação patógeno-hospedeiro. O transportador de cálcio Vcx1 de *C. neoformans* (*vacuolar calcium exchanger 1*) atua na via de sinalização mediada por calcineurina e está envolvido no processo de virulência. Já o fator de transcrição Gat1 regula o metabolismo de nitrogênio em *C. neoformans*. A proteína GRASP (*Golgi reassembly and stacking protein*) está envolvida na secreção de polissacarídeos e virulência em *C. neoformans*.

Com o intuito de organizar os resultados obtidos, os mesmos estão dispostos em três capítulos, os quais correspondem a manuscritos científicos:

Capítulo 1: The vacuolar Ca^{2+} exchanger Vcx1 is involved in calcineurin-dependent Ca^{2+} tolerance and virulence in *Cryptococcus neoformans*

Capítulo 2: The GATA-type transcriptional activator Gat1 regulates nitrogen metabolism in the human pathogen *Cryptococcus neoformans*

Capítulo 3: A role for Golgi reassembly and stacking protein in virulence and polysaccharide secretion in *Cryptococcus neoformans*

2. Objetivos

Considerando que a virulência de microrganismos patogênicos é resultante de um processo de interação complexo e dinâmico que envolve tanto fatores do patógeno quanto do hospedeiro, o objetivo que norteou o desenvolvimento das pesquisas que resultaram na presente Tese é identificar e elucidar a função de genes de *C. neoformans* potencialmente envolvidos nesta interação. Para tanto, as principais metas propostas são:

(i), a construção de mutantes nulos de *C. neoformans* para os genes *VCX1*, *GAT1* e *GRASP*;

(ii), a caracterização fenotípica dos referidos mutantes;

(iii), a avaliação da participação de Vcx1, Gat1 e GRASP na via de sinalização regulada por Ca^{2+}/calcineurina, no metabolismo de nitrogênio e na secreção de polissacarídeos, respectivamente, em *C. neoformans* e,

(iv), a análise funcional dos genes *VCX1*, *GAT1* e *GRASP* de *C. neoformans* no processo de interação patógeno-hospedeiro.

3. Capítulo 1

The Vacuolar Ca^{2+} Exchanger Vcx1 is involved in Calcineurin-Dependent Ca^{2+} Tolerance and Virulence in *Cryptococcus neoformans*.

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The Vacuolar Ca^{2+} Exchanger Vcx1 is involved in Calcineurin-Dependent Ca^{2+} Tolerance and Virulence in *Cryptococcus neoformans*.

Running title: Vacuolar calcium exchanger in *Cryptococcus neoformans*.

Lívia Kmetzsch[1], Charley Christian Staats[1], Elisa Simon[1], Fernanda L. Fonseca[3], Débora L. de Oliveira[3], Luna Sobrino[3], Jéssica Rodrigues[3], Ana Lusia Leal[1], Leonardo Nimrichter[3], Márcio L. Rodrigues[3], Augusto Schrank[1,2], Marilene Henning Vainstein[1,2*]

[1] *Centro de Biotecnologia, Universidade Federal do Rio Grande do Sul, Av. Bento Gonçalves 9500, 43421, Caixa Postal 15005, Porto Alegre, RS 91501-970, Brazil.*

[2] *Departamento de Biologia Molecular e Biotecnologia, Universidade Federal do Rio Grande do Sul, Av. Bento Gonçalves 9500, 43421, Caixa Postal 15005, Porto Alegre, RS 91501-970, Brazil.*

[3] *Laboratório de Estudos Integrados em Bioquímica Microbiana, Instituto de Microbiologia Professor Paulo de Góes, Universidade Federal do Rio de Janeiro, Brazil*.

[*]**Corresponding author.** Mailing address: Centro de Biotecnologia, Universidade Federal do Rio Grande do Sul. Avenida Bento Gonçalves 9500, 43421, Setor 4. Porto Alegre, RS 91501-970, Brazil. Phone: +55 51 3308 6086. Fax: +55 51 3308 7309. **E-mail**: mhv@cbiot.ufrgs.br

Abstract

Cryptococcus neoformans is an encapsulated yeast, that causes a life-threatening meningoencephalitis in immunocompromised individuals. The ability to survive and proliferate at the human body temperature is an essential virulence attribute of this pathogen. This trait is controlled in part by Ca^{2+}-calcineurin pathway, which senses and utilizes cytosolic calcium for signaling. In the present study, the identification of the *C. neoformans* gene *VCX1*, which encodes a vacuolar calcium exchanger, is reported. The *VCX1* knockout results in hypersensitivity to the calcineurin inhibitor cyclosporin A at 35°C, but not at 30°C. Furthermore, high concentrations of $CaCl_2$ lead to growth inhibition of the *vcx1* mutant strain only in the presence of cyclosporin A, indicating that Vcx1 acts in parallel with calcineurin. The loss of *VCX1* does not influence cell wall integrity or capsule size, but decreases secretion of the major capsular polysaccharide glucuronoxylomannan (GXM) in culture supernatants.Vcx1 also influences *C. neoformans* phagocytosis by murine macrophages and is required full virulence in mice. Analysis of cellular distribution by confocal microscopy confirms the vacuolar localization of Vcx1 in *C. neoformans* cells.

Introduction

Calcium (Ca^{2+}) is an intracellular messenger that controls numerous cellular processes. Two essential mediators of calcium signals in eukaryotic cells are the Ca^{2+} binding protein calmodulin and the Ca^{2+}/calmodulin-activated serine/theronine protein phosphatase calcineurin (4, 21). When intracellular calcium levels are low, calcineurin is inactive. An increase in calcium levels in the external environment leads to elevate cytosolic calcium. This process is sensed by calmodulin, which binds the C-terminal region of calcineurin. Then, activated calcineurin prompts transcription of genes whose products allow the cell to survive in stress conditions and maintain calcium homeostasis (3, 5). In mammalian T cells, calcineurin activates the transcription factors NFAT - Nuclear factor of activated T-cells. This pathway is the target of the immunosuppressive drugs cyclosporin A (CsA) and FK506, which inhibit T-cell activation (16).

An *in silico* comparative analysis of fungal Ca^{2+}-signaling components showed that fungi share well conserved key regulators of Ca^{2+}-signaling. These include Ca^{2+}-channels, -pumps, -transporters, -exchangers, calmodulin, calmodulin-dependent kinases and calcineurin. The Ca^{2+}-signaling apparatus of *Saccharomyces cerevisiae* regulates the cell cycle, mating, sensing of glucose starvation, resistance to salt stress and cell survival (41). The stress response in *S. cerevisiae* leads to calcineurin-mediated dephosphorylation and activation of the transcription factor Crz1, which regulates the transcription of more than 160 genes (30, 36). In filamentous fungi *Neurospora crassa* and *Magnaporthe grisea*, there is evidence for the involvement of Ca^{2+} in many physiological

processes, including the cell cycle, sporulation, spore germination, and hyphal growth (41).

The Ca^{2+}/calcineurin signaling pathway in the human pathogenic fungus *Cryptococcus neoformans* has been characterized (reviewed in (19)). In this fungus calcineurin is required for mating, morphogenesis, growth at 37°C and virulence (7, 13, 33). *C. neoformans* calmodulin is essential for viability and acts in response to high temperature by two distinct mechanisms. Only one of these pathways, however, involves Ca^{2+} and calcineurin (22). Furthermore, two important components of the *C. neoformans* Ca^{2+}-signaling network were described (10, 24). The first is Cch1, a Ca^{2+}-permeable channel that mediates calcium entry in *C. neoformans* cells. This plasma membrane calcium channel is required for calcium uptake in low-calcium environments (24). The second is Eca1, a sarcoplasmic/endoplasmic reticulum Ca^{2+}-ATPase that participates in stress tolerance (10). Both components, Cch1 and Eca1, are involved in *C. neoformans* virulence (10, 24), emphasizing the importance of calcium signaling in this pathogen.

Ca^{2+}-exchangers regulate the concentration of cytosolic Ca^{2+} and its transport to storage organelles. This process is achieved by exchanging positive ions across membranes (41). *S. cerevisiae* possess one such previously identified H^{+}/Ca^{2+} -exchanger named Vcx1 (*Sc*Vcx1), which localizes to the vacuolar membrane (9, 32). *Sc*Vcx1 is negatively regulated by calcineurin, acting in Ca^{2+} tolerance and Ca^{2+} sequestration efficiently when calcineurin is inactivated (9). Additionally, *Sc*Vcx1 may also function in Cd^{2+} transport (35). Here we report the identification of the *C. neoformans* gene

VCX1, which encodes a vacuolar calcium exchanger. The *VCX1* knockout results in hypersensitivity to the calcineurin inhibitor cyclosporin A at 35°C, but not at 30°C. Growth analysis of the *vcx1* mutant strain with high concentrations of $CaCl_2$ in the presence or absence of cyclosporin A indicates that Vcx1 confers a much larger degree of calcium tolerance when calcineurin has been inhibited. Importantly, Vcx1 is required for intracellular growth in murine macrophages and for virulence in mice.

Material and Methods

Fungal strains, plasmids and media

C. neoformans serotype A H99 strain was recipient for construction of the mutant strain. Strains were maintained on YPD medium (1% yeast extract, 2% peptone, 2 % dextrose, and 1.5% agar). YPD plates with hygromycin (200 µg/ml) were used to select *C. neoformans VCX1* deletion transformants (*vcx1* strain). YPD plates added of nourseothricin (100 µg/ml) were used to select *C. neoformans VCX1* complementation transformants (*vcx1*::*VCX1* strain). Plasmid pJAF15 (14) was the source of a hygromycin resistance cassette and pAI4 (17) the source of a nourseothricin resistance cassette. Plasmids were maintained in *Escherichia coli* grown at 37°C in LB broth or on agar supplemented with 50 µg/ml of kanamycin.

In silico analysis of the *C. neoformans VCX1* ortholog

The putative *C. neoformans VCX1* gene sequence was identified by BLAST search of the *C. neoformans* var. *grubii* strain H99 genomic database at the Broad Institute using the Vcx1 sequence of *S. cerevisiae* (GenBank

accession number NP_010155.1). The amino acid sequences of Vcx1 orthologs from *S. cerevisiae*, *Candida albicans, Aspergillus fumigatus, Schizosaccharomyces pombe, Coprinus cinereus, Ustilago maydis, Arabidopsis thaliana, Oryza sativa* and *C. neoformans* were aligned using ClustalX2 (23). Mega4 was utilized for phylogenetic analysis applying the Neighbor-Joining method and the tree architecture was inferred from 1,000 bootstraps (37). Search for conserved domains in the ortholog proteins was performed using Pfam database (http://pfam.sanger.ac.uk/). Prediction of putative transmembrane segments was conducted by the TMHMM server (http://www.cbs.dtu.dk/services/TMHMM/).

Disruption and complementation of *C. neoformans VCX1*

Disruption of *VCX1* was achieved with DNA constructs generated by the Delsgate methodology (15). A Gateway cloning system donor vector (Invitrogen) carrying the hygromycin selectable marker for *C. neoformans* transformation was constructed. A 2.2 Kb PCR product encompassing the hygromycin marker cassette was amplified from pJAF15 plasmid and cloned into the EcoRV site of pDONR201 (Invitrogen) resulting in a vector named pDONRHYG. The 5' and 3' *VCX1* flanks (900 bp each) were PCR amplified, and gel purified using Illustra GFX PCR DNA and Gel Band Purification kit (GE Healthcare). Approximately 300 ng of vector pDONRHYG and 30 ng of each PCR product were submitted to BP clonase reaction, according to manufacturer's instructions (Invitrogen). This reaction was transformed into *E. coli* OmniMAX 2-T1 cells. After confirmation of the correct deletion construct, the plasmid was linearized by I-SceI digestion prior to *C. neoformans* biolistic

transformation (38). The transformants were screened by colony PCR, and the deletion was confirmed by Southern blot analysis and semi-quantitative RT-PCR. For complementation of the mutation, a 4.4 Kb genomic PCR fragment carrying the wild-type *VCX1* gene was cloned into the SmaI site of vector pAI4. The resulting plasmid was used for transformation of the *vcx1* mutant strain and the transformants were selected in the presence of nourseothricin (200 µg/ml). Random genomic insertion of the complemented gene was confirmed by Southern blot analysis and semi-quantitative RT-PCR. The primers used in these constructions are listed in Table S1.

Phenotypic characterization assays

For phenotypic characterization, wild type (WT), *vcx1* mutant and complemented strains were grown on YPD medium for 16 hours, washed and adjusted to a cell density of 10^7 cells/ml. The cell suspensions were serially diluted 10-fold and 3 µl of each dilution was spotted onto YPD agar supplemented with $CaCl_2$ (50, 100, 150 or 200 mM), $MnCl_2$ (2, 4, 6 or 8 mM), $CdCl_2$ (20 or 50 µM), and/or cyclosporin A (100 µg/ml). The plates were incubated for 2 days at 30°C, 35°C or 37°C and photographed. Fungal cells were also grown at 37°C in 5% CO_2, in YPD medium with alkaline, neutral or acidic pHs (8.5, 7, and 4, respectively), or in the presence of Congo Red (0.5%). Melanin production was examined on niger seed agar plates containing 0.1% glucose. Capsule formation was examined by microscopy after incubation for 24 hours at 30°C in a minimal medium (12) and staining with India ink. Relative capsule sizes were defined as the distance between the cell wall and the capsule outer border by cell diameter. ImageJ software was utilized to

determine capsule measurements of one hundred cells of each strain. The content of extracellular GXM in these culture supernatants was determined by ELISA according to a protocol previously described (11). The content of extracellular GXM in culture supernatants in the presence of cyclosporin A (25 up to 150 μg/ml) was also determined. Phenotypic analysis of the cell surface architecture was performed after incubation of yeast cells with the wheat germ lectin (WGA), calcofluor white, and the monoclonal antibody 18B7 (12). These probes were used to visualize, by fluorescence microscopy, the surface distribution of N-acetylglucosamine (GlcNAc) oligomers (WGA), cell wall chitin (calcofluor) and GXM (18B7), following a previously described protocol (12).

Virulence assay

Virulence studies were conducted according to a previously described intranasal inhalation infection model (6) using eight female BALB/c mice (approximately 5 weeks old) for each strain tested. Fungal cells were cultured in 50 ml of YPD medium at 30°C overnight with shaking, washed twice and re-suspended in PBS. Mice were infected with 10^7 yeast cells suspended in 50 μl PBS and daily monitored. Kaplan–Meier analysis of survival was performed using GraphPad Prism Software. Animal studies were approved by the Federal University of Rio Grande do Sul Ethics Committee.

Macrophage infection assay

The susceptibility of fungal cells to the antifungal action of phagocytes was determined by counting of colony forming units (CFU) after interaction of WT, *vcx1* mutant and *vcx1*::*VCX1* complemented strains with the murine

macrophage-like cell line RAW 264.7. Murine cells were maintained at 37°C in 5% CO_2 in Dulbecco's modified Eagle's medium (DMEM) supplemented with 5% heat-inactivated fetal bovine serum (FBS). Fungal cells were opsonized with monoclonal antibody 18B7 (1 µg/ml). Macrophages were seeded at a concentration of 10^5cells/well in a 96-well cell culture plate and incubated overnight. Then, 10^6 fungal cells were inoculated in each well, and after 1 h the wells were washed to remove unattached, extracellular fungal cells. After 20 h of incubation, infected cultures were again washed and sterile ice-cold dH_2O was added to each well to promote macrophage lysis. Fungal viability was measured by plating the lysates on YPD for CFU determination after cultivation of the plates for 48 h at 30°C. The assay was performed in triplicate sets for each strain. Student's *t* test was used to determine the statistical significance of differences in fungal survival. To determine the rate of phagocytosis of WT, *vcx1* mutant and complemented cells during interaction with RAW 264.7 cells, yeast cells were incubated with FITC at 0.5 mg/ml in PBS for 10 min at room temperature. Then, the cells were washed with PBS and incubated with macrophage-like cells for 1 h at a 5:1 fungi-host cell ratio, followed by extensive washing with PBS for removal of non-adherent fungi. The fluorescence intensity of the macrophage-like cells was therefore a function of association with FITC-labeled *C. neoformans*. The infected cells were then detached from tissue culture plates by scrapping, fixed with 4% paraformaldehyde and analyzed in a FACScalibur (BD Biosciences). The data were analyzed using the winMDI 2.9 software.

Construction of *C. neoformans* Vcx1-mCherry fusion strain

In order to assess the subcellular localization of Vcx1 in *C. neoformans* cells, a cassette carrying a Vcx1-mCherry fusion was constructed. Briefly, a 2Kb fragment encompassing the *VCX1* promoter and coding sequences without the STOP codon was amplified using genomic DNA from H99 strain as template. The pLKB25 plasmid (18) was utilized as a template to amplify a second fragment containing mCherry-GPD1 terminator and the NEO resistance gene. These fragments, which overlap by ~40bp, were combined and utilized as template for the overlap PCR. The product of the overlap PCR was cloned into pCRTOPO2.1 vector. This final construct was transformed in *C. neoformans* *vcx1* mutant strain by biolistic transformation (38). Transformants (Vcx1-mCherry strains) were selected in YPD plates added of 200 µg/ml of neomycin. These cells were visualized and photographed using an Olympus FluoView 1000 Confocal Laser Scanning Microscope.

Quantitative real time RT-PCR analysis

For RNA extraction, cultures of WT and *vcx1* mutant cells were grown overnight in YPD medium at 37°C with shaking. Three independent sets of RNA samples from independent experiments were prepared using Trizol reagent (Invitrogen) according to the manufacturer's protocol. After DNase treatment, RNA preparations were purified using RNAeasy mini columns (Qiagen), and reverse transcriptase reactions were performed. Real-time PCR reactions were performed in an Applied Biosystems 7500 Real-Time PCR System. PCR

thermal cycling conditions were an initial step at 95 °C for 5 min followed by 40 cycles at 95 °C for 15 s, 60 °C for 20 s and 72 °C for 20 s. Platinum SYBR green qPCR Supermix (Invitrogen) was used as reaction mix, supplemented with 5 pmol of each primer and 2 µl of the cDNA template in a final volume of 25 µl. All experiments were done in three independent cultures and each cDNA sample was analyzed in duplicate with each primer pair. Melting curve analysis was performed at the end of the reaction to confirm a single PCR product. Data was normalized to actin cDNAs amplified in each set of PCR experiments. Relative expression was determined by the $2^{-\Delta CT}$ method (25). The primers utilized in these experiments are listed in Table S1.

Determination of relative levels of intracellular calcium concentration

The relative intracellular free calcium concentration was determined using acetoxymethylester of Fura-2 (Fura 2-AM, Invitrogen). Briefly, WT and *vcx1* mutant cells were cultured in YPD medium overnight with shaking. Then, 10^7 cells of each strain were incubated for 1 h in fresh YPD amended or not with 100mM $CaCl_2$. The cells were washed three times with PBS and loaded with 10µM of Fura 2-AM for 30 min at 37°C. After extensive washing, Fura-2 fluorescence was measured by alternating the excitation wavelengths at 340 and 380 nm with emission wavelength fixed at 505 nm. The relative intracellular calcium concentration is expressed by the ratio between fluorescence intensities with excitation wavelengths at 340 and 380 nm. All data presented are representative of three independent experiments.

Results

Identification of the vacuolar calcium exchanger Vcx1 ortholog in *C. neoformans*

The *VCX1* gene was identified in the *C. neoformans* var. *grubii* H99 genomic database at the Broad Institute (accession number CNAG_00025.1) (http://www.broadinstitute.org/annotation/genome/cryptococcus_neoformans/MultiHome.html) based on its similarity to the vacuolar calcium exchanger Vcx1 from *S. cerevisiae*. The *C. neoformans VCX1* coding region is 2,399 bp long, contains nine introns and encodes a putative 604-amino-acid protein. Ca^{2+}-exchangers proteins are characterized by the presence of calcium/hydrogen antiporter (*ChaA*, COG0387), calcium/proton exchanger (*caca2*, TIGR00846; *cax*, TIGR00378), and sodium/calcium exchanger superfamily (*Na_Ca_ex*, pfam01699) domains (41). BLAST search using the Conserved Domain Database at NCBI (http://www.ncbi.nlm.nih.gov/structure/cdd/cdd.shtml) revealed that these domains are also present in the *C. neoformans* Vcx1 ortholog. Furthermore, a phylogenetic analysis including Vcx1 sequences from distinct eukaryotic organisms was performed (Figure 1A). *C. neoformans* Vcx1 has higher similarity to Vcx1 of *U. maydis* (36 % identity and 52 % similarity) and lowest similarity to that in *A. thaliana* (21 % identity and 39 % similarity) when the amino acids of each protein were compared. The phylogeny tree splits into two clades, corresponding to fungal and plant Vcx1 orthologs. As expected, *C. neoformans* Vcx1 clusters with other basidiomycete fungal Vcx1 proteins. The domain architecture of the *C. neoformans* Vcx1 was compared to the orthologs herein analyzed. All of them possess two well conserved domains

related to sodium/calcium exchanger superfamily (*Na_Ca_ex*, pfam01699). Moreover, prediction of transmembrane (TM) regions revealed that all the Vcx1 proteins analyzed had ten or eleven predicted TM domains (Figure 1B), in agreement with known Ca^{2+} exchangers (41).

The *vcx1* mutant displayed calcineurin-dependent Ca^{2+} sensitivity

In order to investigate the functional role of *VCX1* in *C. neoformans*, knockout and complemented strains were constructed. Deletion and complementation of *VCX1* were confirmed by Southern blot analysis and semi-quantitative RT-PCR (Figure S1). The ability to grow at the human body temperature (36° - 37°C) is one of the most important virulence factors of *C. neoformans* (28). This trait is controlled in part by Ca^{2+}-calcineurin pathway, which senses and utilizes cytosolic calcium for signaling (19). We then asked whether the *C. neoformans* Vcx1 participates in this signaling pathway during high temperature growth. Our results demonstrate that Vcx1 is required for *C. neoformans* growth at 35°C (semi-permissive temperature) only in the presence of cyclosporin A, a calcineurin inhibitor (Figure 2A). Furthermore, the role of *VCX1* in Ca^{2+} tolerance was evaluated by monitoring growth of *vcx1* mutant strain in YPD agar plates supplemented with increasing concentrations of $CaCl_2$ amended or not with cyclosporin A (Figure 2 B and C). The growth of the *vcx1* mutant strain was inhibited only in the presence of cyclosporin A, indicating that Vcx1 confers tolerance to a wider range concentration of calcium when calcineurin was inhibited (Figure 2C). Together, these results suggest that Vcx1 influences Ca^{2+}-calcineurin signaling pathway in *C. neoformans*. Since *S. cerevisiae* calcineurin regulates the transport of cations by variants of Vcx1

(35), we evaluated the Cd^{2+} and Mn^{2+} tolerance of *C. neoformans vcx1* mutant strain. No differences on fungal growth were observed under conditions of high concentrations of $CdCl_2$ or $MnCl_2$ (Figure 2D). However, when calcineurin was inhibited by cyclosporin A, the *vcx1* mutant strain exhibited a slightly-increased resistance against high concentration of $CdCl_2$ when compared to WT and complemented strains (Figure 2E).

Disruption of *VCX1* does not influence capsule size, but decreases extracellular GXM secretion

The *vcx1* mutant strain was tested for its ability to grow at 37°C, for capsule size and for melanin production since these traits are thought to be the major virulence factors in *C. neoformans* (31). The *VCX1* knockout apparently did not interfere with any of these features, when compared to WT cells (Figure 3 and data not shown). Moreover, the deletion of *VCX1* also did not cause defects in cell wall integrity, as assessed by staining of chitin and GlcNAc oligomers (Figure 3A), and by a spot growth assay on plates containing Congo Red (Figure S2). However, mutant cells showed an impaired ability to produce extracellular GXM, as concluded from the lower polysaccharide contents in culture supernatants of the *vcx1* mutant strain, in comparison to WT and complemented cells ($P < 0.05$) (Figure 3C). We believe that the antibody-based lower GXM detection in supernatants of *vcx1* mutant cells reflects a decreased production of this extracellular polysaccharide rather than the production of a structurally modified GXM, since the surface polysaccharide was regularly recognized by mAb 18B7. This observation, which echoes previous findings with a secretion mutant of *C. neoformans* (34), suggests a role for Vcx1 on the

release of capsular polysaccharides to the extracellular environment. The levels of extracellular GXM contents in culture supernatants of WT, *vcx1* mutant and complemented cells treated with cyclosporin A were also quantified. Different concentrations of cyclosporin A (ranging from 25 to 150 μg/ml) completely inhibited GXM secretion by all strains tested (Figure 3D).

VCX1 influences *C. neoformans* phagocytosis by macrophages and is required for full virulence in animal cryptococcosis

The susceptibility of WT, *vcx1* mutant and *vcx1*::*VCX1* complemented strains to the antimicrobial action of macrophage-like cells was evaluated *in vitro*. In comparison to WT and complemented cells, the *vcx1* deletion mutant showed decreased rates of survival after interaction with the phagocytes (Figure 4A, $P < 0.05$). To exclude the possibility that the lower rate of *vcx1* mutant survival inside macrophages was due to its hypersensitivity to extreme pH or to 5% CO_2, fungal cells were grown in YPD medium with alkaline, neutral or acidic pH (8.5, 7, and 4, respectively) or in 5% CO_2. However, no growth differences were observed in these conditions (Figure S2). The lower CFU counts for the *vcx1* mutant could be due to a decreased survival within the macrophages (lower viability or slower proliferation within the macrophages) or, alternatively, it may be due to a lower rate of phagocytosis. To test this hypothesis, the rates of phagocytosis of WT, *vcx1* mutant and complemented cells were measured by flow cytometry. In fact, the rate of phagocytosis of the *vcx1* mutant cells was significantly lower when compared to WT and complemented cells (Figure 4B), indicating that *VCX1* is required for efficient phagocytosis and perhaps has some minor role in survival within the macrophages.

These observations encouraged us to test the role of Vcx1 in the pathogenesis of *C. neoformans* in a mouse inhalation model of cryptococcosis. Mice inoculated with WT and complemented strains had mean survival times of four and five days, respectively. Statistical analysis revealed no significant difference between mortality rates caused by these two strains (P=0.79). In contrast, mice inoculated with *vcx1* mutant cells survived longer, with a mean survival time of 9.5 days (P=0.002) (Figure 4C). This result demonstrates that Vcx1 is required for the virulence of *C. neoformans* in animal cryptococcosis.

Vcx1 localizes to the vacuole of *C. neoformans* cells

In order to confirm the predicted vacuolar localization of Vcx1 in *C. neoformans* cells, a mCherry tagged Vcx1 strain was constructed. Figure 5 illustrates the co-localization of mCherry tagged Vcx1 with vacuole organelles, which appear as a depression in the DIC image. This result confirms that Vcx1 localizes to the vacuole of *C. neoformans* cells.

Disruption of *VCX1* influences the expression of other calcium transporters in *C. neoformans*

Since *C. neoformans* Cch1 and Eca1 are also calcineurin-pathway calcium transporters, the transcript levels of these genes were evaluated in WT and *vcx1* mutant strains. Disruption of *C. neoformans VCX1* led to a decrease in expression of *ECA1*, but the *CCH1* transcription level was not influenced. Furthermore, we also analyzed the expression of two putative orthologs of well characterized calcium transporters of *S. cerevisiae*, *PMC1*, a vacuolar calcium

ATPase, and *PMR1*, a Golgi calcium ATPase (8, 29). Interestingly, the transcript levels of the *C. neoformans PMC1* ortholog significantly increased in *vcx1* mutant cells ($P < 0.05$). A slight increase in *PMR1* ortholog expression was also observed in *vcx1* mutant cells (Figure 6).

Loss of *VCX1* influences the relative level of intracellular calcium concentration in *C. neoformans* cells

In order to determine the relative concentration of free calcium in WT and *vcx1* mutant strains the calcium sensitive dye Fura 2-AM was utilized. This fluorescent calcium indicator can passively diffuse across cell membranes. Once inside the cell, the esters are cleaved by intracellular esterases to yield cell-impermeant fluorescent indicators. Upon binding Ca^{2+}, Fura-2 exhibits an absorption shift from 380 to 340 nm of excitation. Therefore, the relative Ca^{2+} concentration was obtained based on the fluorescence ratio after dual-wavelength excitation. The *vcx1* mutant strain has an increased relative level of intracellular calcium concentration when compared to WT strain (Figure 7). This increase is more pronounced when an excessive amount of $CaCl_2$ was added to the medium, indicating that the loss of *VCX1* influences the relative level of intracellular calcium concentration. This phenotype is probably due to a calcium transport defect to vacuoles in *vcx1* mutant strain, since Ca^{2+}-exchangers regulate the concentration of cytosolic Ca^{2+} and its transport to storage organelles (41).

Discussion

The calcium-calcineurin signaling pathway in *C. neoformans* is fundamental to sense and to adapt to the human host *millieu*. In addition to its importance for high temperature growth, calcineurin is also essential for cell wall integrity, mating and monokaryotic fruiting (7, 13, 19, 20, 28, 33). In the present study Vcx1, a new component of the *C. neoformans* Ca^{2+} signaling network, was identified. This vacuolar calcium exchanger is part of a conserved family of Ca^{2+}-exchangers that regulate cytosolic calcium concentration and transport into Ca^{2+}-storage organelles (i.e. vacuoles) in distinct eukaryotes (41).

Firstly, we investigated if *C. neoformans* Vcx1 acts in the Ca^{2+}-calcineurin pathway during host temperature growth. In fact, Vcx1 is required for *C. neoformans* growth at 35°C only when calcineurin had been inhibited. Moreover, Vcx1 participates in Ca^{2+} tolerance in a calcineurin-dependent manner. These findings suggest that Vcx1 influences Ca^{2+}-calcineurin signaling pathway in *C. neoformans*, in agreement with the *S. cerevisiae* Vcx1 ortholog (9, 35). Calcineurin significantly affects Vcx1 Ca^{2+}/H^{+} exchange activity in *S. cerevisiae*. The negative regulation of *Sc*Vcx1 by calcineurin is thought to be post-translational and independent of the transcription factor Crz1, which regulates genes encoding ion pumps and cell wall biosynthetic enzymes (9, 30, 36). No ortholog of the *CRZ1* gene has been identified in *C. neoformans*. It is possible that *C. neoformans* contains different or more than one transcription factor responsive to calcineurin (19).

The loss of *C. neoformans* Vcx1 by knockout did not cause defects in cell wall integrity, melanin production and capsule size, but the *vcx1* mutant showed a reduced ability to produce extracellular GXM. Although the concentration of

extracellular GXM is usually related to capsule enlargement, Panepinto and colleagues recently demonstrated that a *sec6* mutant of *C. neoformans* produces a normal capsule even under conditions in which GXM secretion is decreased (34). Our result suggests that, although yeast cells lacking Vcx1 expression are able to assemble a normal capsule, the protein is somehow required for polysaccharide release.

A key feature of cryptococcal pathogenesis is its ability to survive and replicate inside macrophages, in a process that requires GXM release for further accumulation in cytoplasmic vesicles (39). GXM is toxic for the macrophages, which implies that polysaccharide secretion reduces the antimicrobial activity of phagocytes. Furthermore, *C. neoformans* possess mechanisms that allow cell-to-cell spread and extrusion from infected macrophages (1, 2, 26, 27, 39, 40). These processes supposedly require production of GXM. Therefore, capsular polysaccharide interferes with macrophage function at multiple levels (reviewed in (40)). In this context, the defective ability to produce extracellular GXM that was observed in *vcx1* mutant could be related to its reduced rate of phagocytosis and survival after macrophage infection.

In our study, Vcx1 was required for full virulence during infection. This same phenotype was observed for *C. neoformans* mutants lacking expression of Cch1 and Eca1, a plasma membrane calcium channel and a sarcoplasmic/endoplasmic reticulum Ca^{2+}-ATPase, respectively (10, 24). These results emphasize the key role of calcium signaling for cryptococcal virulence. Calcineurin is essential for *C. neoformans* survival in the host environment (19,

33). Our results showed that in the presence of the calcineurin inhibitor cyclosporin A, *vcx1* mutant failed to growth at high temperature, suggesting that Vcx1 acts in parallel with calcineurin in response to the host's temperature. *C. neoformans* Vcx1 also influences the expression of other calcium transporters. The transcript level of *PMC1* ortholog, a putative vacuolar calcium ATPase, was significantly increased in *vcx1* mutant when compared to WT strain, probably due to a compensatory effect, since *PMC1* also transport calcium into vacuoles (8).

In conclusion, we have shown that Vcx1, a vacuolar calcium transporter, influences *C. neoformans* phagocytosis by macrophages and is required for full virulence in animal infection. Additionally, Vcx1 is involved in calcineurin-dependent Ca^{2+} tolerance, acts in Ca^{2+}-calcineurin signaling pathway in *C. neoformans,* and influences the relative intracellular calcium concentration. Further studies are necessary to address the role of Vcx1 in the release of GXM to the extracellular environment, and to completely understand how this component of the Ca^{2+}-calcineurin pathway contributes to the pathogenesis of cryptococcosis.

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pAI4 and pLKB25 plasmids and Dr. Arturo Casadevall for providing the monoclonal antibody anti-GXM (18B7). The authors also thank the Electron Microscopy Center of the Federal University of Rio Grande do Sul (CME, UFRGS, Brazil) for the confocal microscopy analysis and Henrique Biehl for technical assistance. Automated DNA sequencing was performed at the facilities of the Brazilian Genome Network at the Center of Biotechnology, CBiot-UFRGS-RS.

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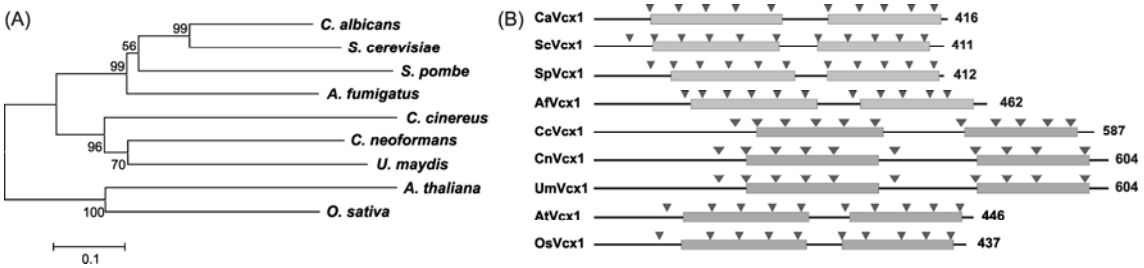

Fig. 1. ***In silico*** **analysis of the** ***C. neoformans*** **Vcx1 ortholog. A.** Phylogenetic analysis applying the Neighbor-Joining method including Vcx1 sequences from distinct eukaryotic organisms, as follows: *S. cerevisiae* (accession number at NCBI NP_010155.1), *C. albicans* (accession number at NCBI XP_711893.1), *A. fumigatus* (accession number at NCBI XP_750174.2), *S. pombe* (accession number at NCBI O59768), *C. cinereus* (accession number at Broad Institute CC1G_02539), *U. maydis* (accession number at Broad Institute UM_05132), *A. thaliana* (accession number at NCBI Q945S5), *O. sativa* (accession number at NCBI NP_00105030.1) and *C. neoformans* (accession number at Broad Institute CNAG_00025.1). The bar marker indicates the genetic distance, which is proportional to the number of amino acid substitutions. Bootstrap values obtained with 1,000 resamplings are shown at the nodes. B. Domain architecture in the Vcx1 orthologs of different eukaryotic organisms. The sodium/calcium exchanger superfamily (*Na_Ca_ex*, pfam01699) domain, a characteristic domain in Ca^{2+} exchangers, is represented by gray boxes. The transmembrane domains are represented by arrowheads. The lengths of each sequence protein (in amino acids) are indicated in the right.

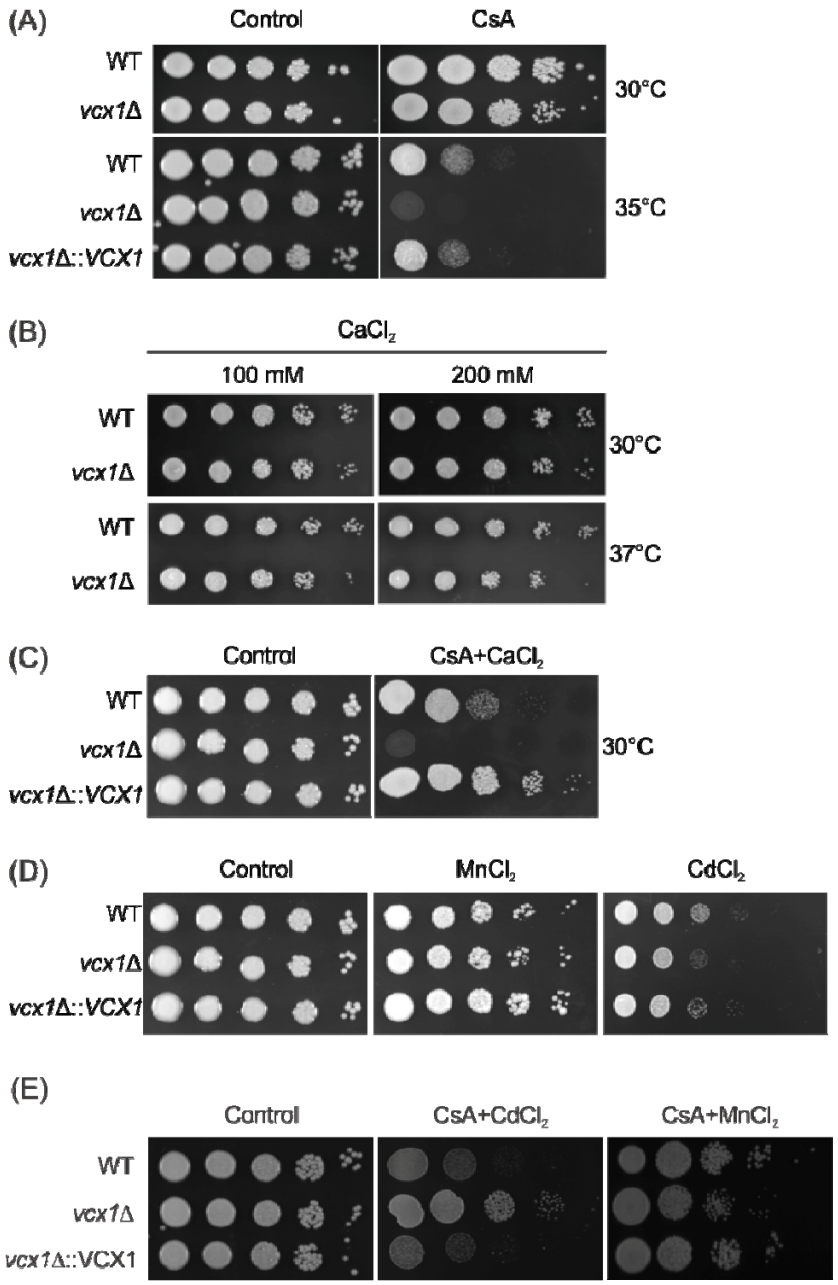

Fig. 2. **The *vcx1* mutant displays calcineurin-dependent Ca^{2+} sensitivity.** Ten-fold serial dilutions of wild-type H99 (WT), *vcx1* mutant (*vcx1Δ*) and *vcx1::VCX1* complemented (*vcx1Δ::VCX1*) cells were plated in YPD agar containing 100μg/ml of cyclosporin A (CsA) (A), 100 or 200mM of $CaCl_2$ (B), 50mM of $CaCl_2$ amended with 100μg/ml of CsA (C), 4mM of $MnCl_2$ or 50μM of $CdCl_2$ (D), and 4mM of $MnCl_2$ or 50μM of $CdCl_2$ amended with 100μg/ml of CsA

(E). The plates were incubated for 2 days at 30°C, 35°C or 37°C, as indicated. As control, cells were grown in YPD agar only.

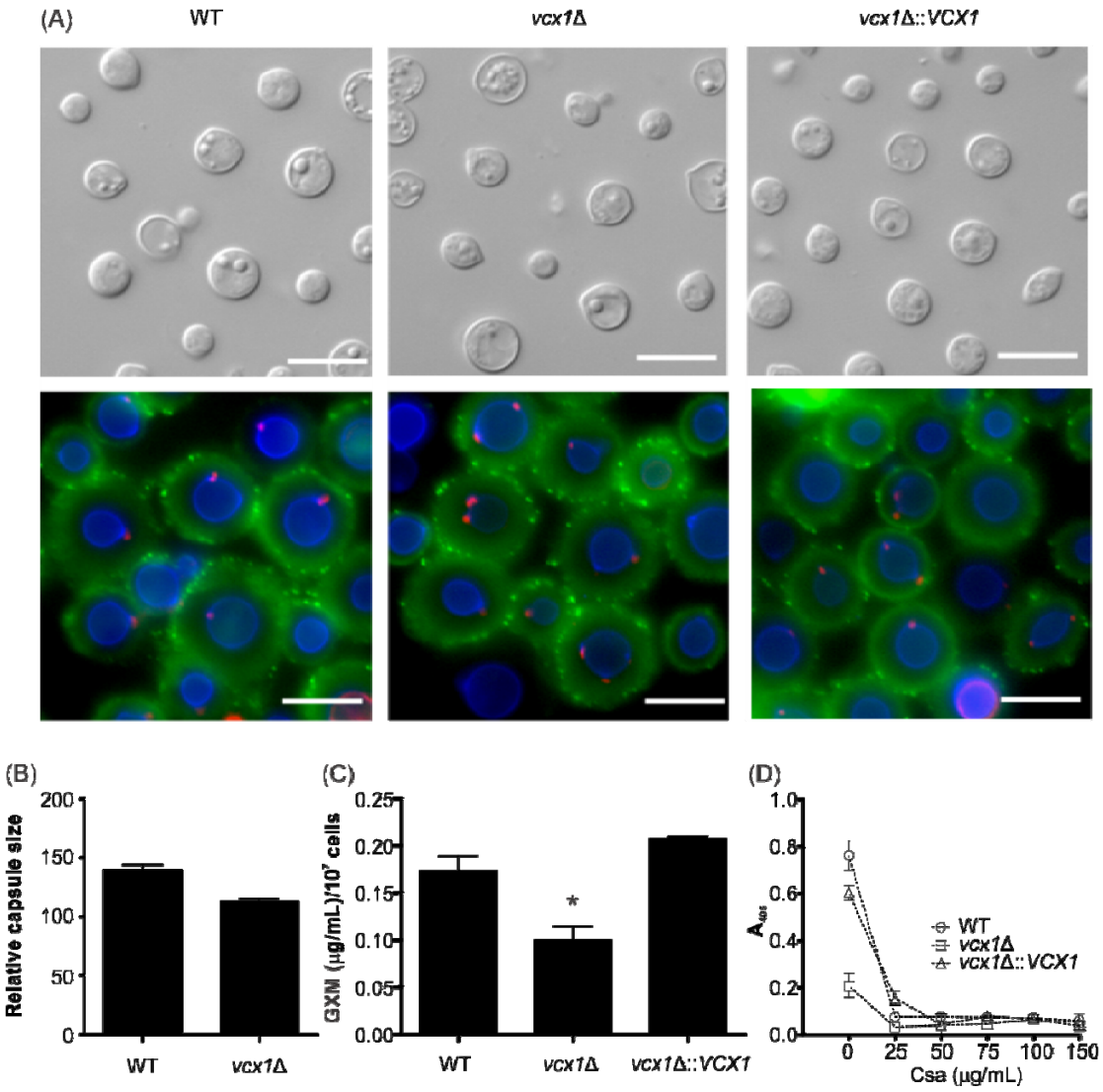

Fig.3. **Disruption of *VCX1* does not influence capsule size, but decreases extracellular GXM secretion. A.** Fluorescence microscopy of WT, *vcx1* mutant and *vcx1::VCX1* complemented mutant cells stained with the lectin WGA (red spots), calcofluor white (blue staining), and the monoclonal antibody 18B7 (green), to visualize GlcNAc oligomers, cell wall and GXM, respectively. Scale bar, 10µm. **B**. Relative capsule size of WT and *vcx1* mutant cells. **C**. Content of extracellular GXM in culture supernatants of WT, *vcx1* mutant and *vcx1::VCX1* complemented mutant cells, determined by ELISA. *, $P < 0.05$. **D.** Content of extracellular GXM in culture supernatants of WT, *vcx1* mutant and *vcx1::VCX1*

complemented mutant cells incubated with different concentrations of Cyclosporin A (CsA), determined by ELISA.

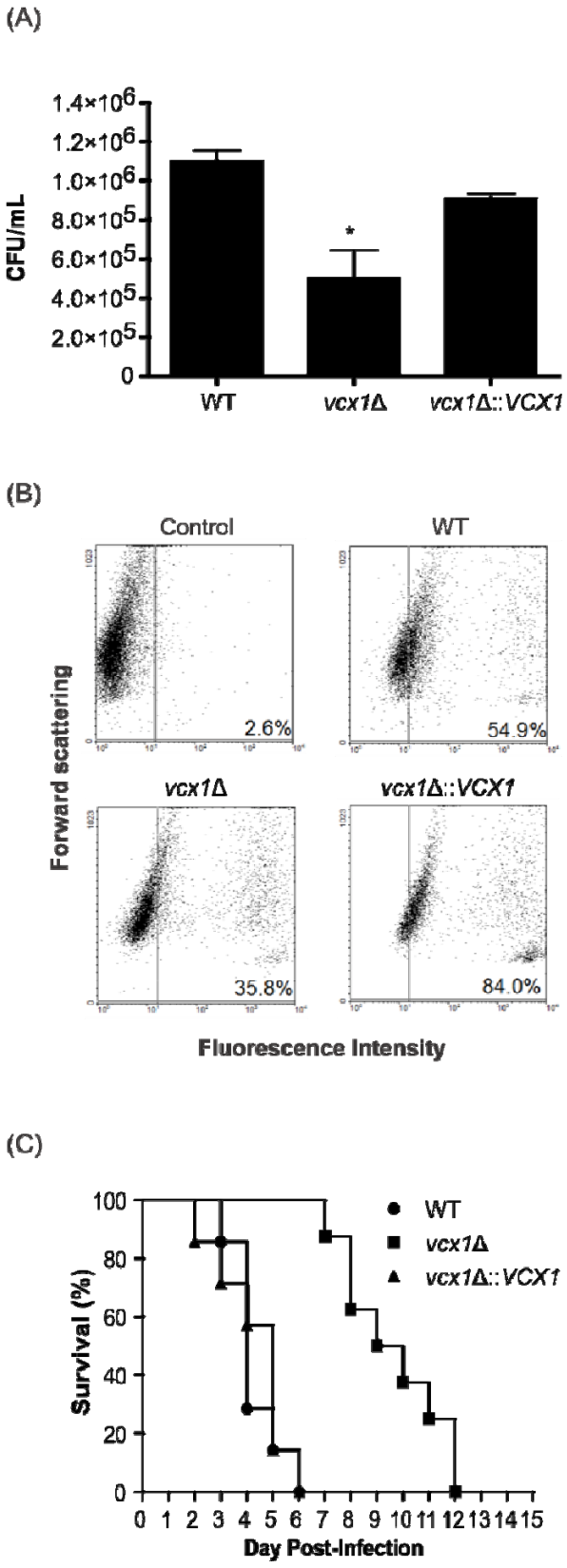

Fig. 4. *VCX1* influences *C. neoformans* phagocytosis by macrophages and is required for full virulence in mice. A. CFU counting after macrophage

infection with WT, *vcx1* mutant and *vcx1*::*VCX1* complemented strains. *, $P < 0.05$. **B**. Rates of phagocytosis of WT, *vcx1* mutant and complemented cells measured by flow cytometry. **C.** Virulence assay of WT, *vcx1* mutant and *vcx1*::*VCX1* complemented strains in an intranasal inhalation infection model using BALB/c mice.

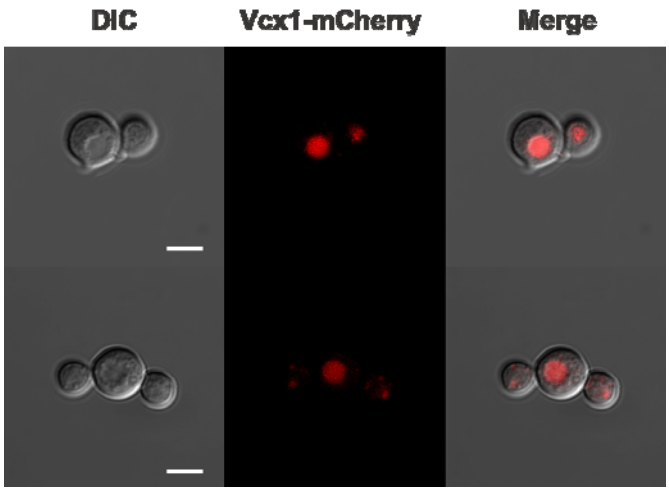

Fig. 5. **Vcx1 localizes to the vacuoles of *C. neoformans* cells.** Confocal microscopy of Vcx1-mCherry fusion strain cells. DIC represents differential interference contrast. The mCherry tagged Vcx1 (red) co-localizes with vacuole organelles, which appear as a depression in the DIC image. Scale bar, 5μm.

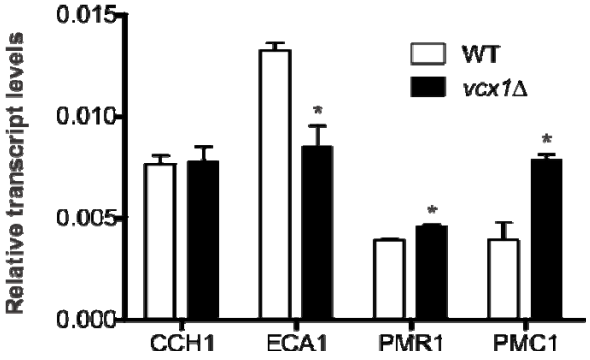

Fig. 6. **Disruption of *VCX1* influences the expression of other calcium transporters in *C. neoformans.*** The relative expression of different *C. neoformans* calcium transporters (*CCH1*, *ECA1*, *PMR1,* and *PMC1*) in WT and *vcx1* mutant cells was quantified by qRT-PCR. The measured quantity of the mRNA in each of the samples was normalized using the *Ct* values obtained for the actin gene. The access numbers for the *C. neoformans* orthologs of *PMR1* and *PMC1* at the Broad Institute are CNAG_05135.2 and CNAG_01232.2, respectively. Data are shown as mean ± SD. *, $P < 0.05$.

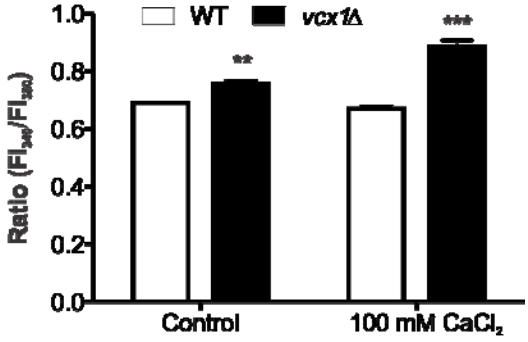

Fig. 7. **Disruption of *VCX1* influences the relative levels of intracellular calcium concentration in *C. neoformans.*** The relative levels of intracellular calcium concentration in WT and *vcx1* mutant cells were determined using the calcium sensitive dye Fura 2-AM. The relative Ca^{2+} concentration was obtained based on the fluorescence ratio after dual-wavelength excitation (FI_{340}/FI_{380}). FI, fluorescence intensity. Control, fresh YPD medium. Data are shown as mean ± SD. **, $P < 0.05$, ***, $P< 0.01$.

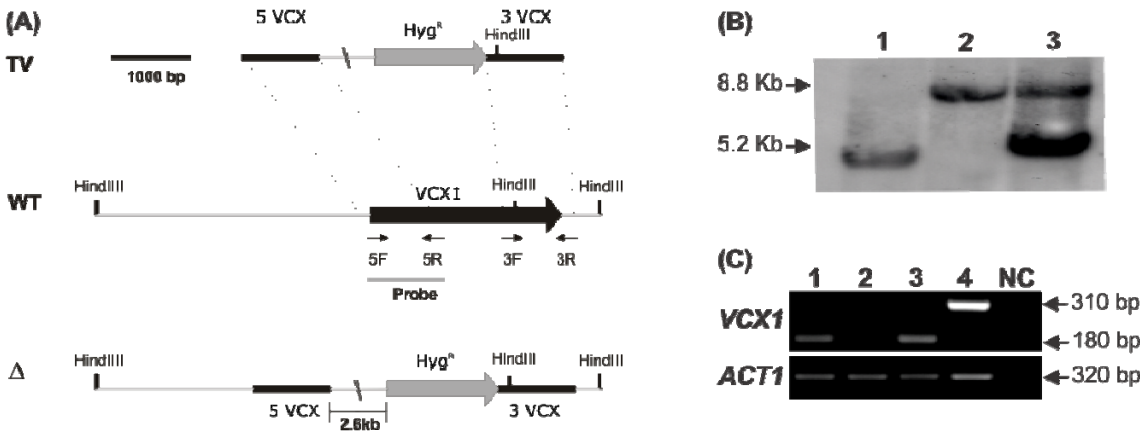

Figure S1. Disruption and complementation of *C. neoformans VCX1.* A. Vcx1 deletion scheme. TV represents the targeting vector constructed by Delsgate metodology. 5 VCX and 3 VCX represent the 5' and 3' gene flanks of Vcx1 gene, respectively. 5F and 5R: primers utilized to amplify 5' flank of Vcx1 gene. 3F and 3R: primers utilized to amplify 3' flank of Vcx1 gene. Hyg^R: cassette that confers hygromicin resistance. WT represents the wild type locus of the *VCX1* gene in H99 strain. Δ represents the *VCX1* locus in the *vcx1* mutant strain. The cleavage sites of HindIII restriction enzyme are indicated in the deletion scheme. **B**. Confirmation by Southern blot. Genomic DNA (10µg) from WT (lane 1), *vcx1* mutant (lane 2) and *vcx1::VCX1* complemented (lane 3) strains were digested with HindIII restriction enzyme. The 5' gene flank was used as probe in Southern hybridization. Left numbers (in base pairs) indicate the hybridization signals size based upon the position of molecular size marker. **C**. Semi-quantitative RT-PCR using cDNA from WT (lane 1), *vcx1* mutant (lane 2) and *vcx1::VCX1* complemented (lane 3) strains as template. Right numbers (in bp) indicate the length of the transcripts amplification for *VCX1* (upper panel) and *ACT1* (lower panel) genes. Lane 4: positive control using genomic DNA as template. NC: negative control of the PCR reaction.

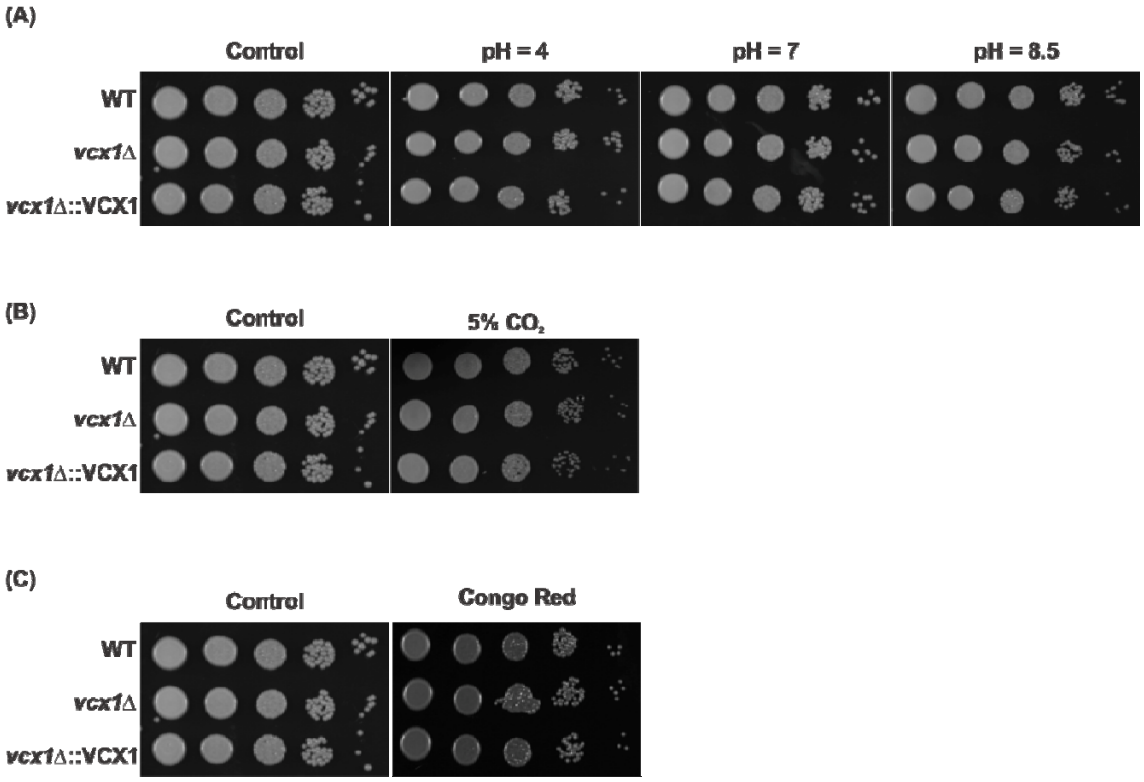

Fig. S2. Ten-fold serial dilutions of wild-type H99 (WT), *vcx1* mutant (*vcx1Δ*) and *vcx1::VCX1* complemented (*vcx1Δ::VCX1*) cells were plated in YPD agar in alkaline, neutral and acidic pH (A), in 5% CO_2 (B), and with 0.5% Congo Red (C).

Table S1. **List of primers used in this study.**

Primer name	Sequence (5'-3')	Purpose
CnVcx5F	AAAATAGGGATAACAGGGTAATAACAA ACCATGTTTCACCAAAGC	Disruption construct for *VCX1*, 5' flank
CnVcx5R	GGGGACAAGTTTGTACAAAAAAGCAGG CTATACAAGAAGGATGTTACTGAG	Disruption construct for *VCX1*, 5' flank
CnVcx3F	GGGGACCACTTTGTACAAGAAAGCTG GGTAGGTTTAATCTTGCTTCCTAT	Disruption construct for *VCX1*, 3' flank
CnVcx3R	AAAAATTACCCTGTTATCCCTAAAAGTG GTATAAAGAAGCTCAGGG	Disruption construct for *VCX1*, 3' flank
VcxcompF	TTTGTTGGTGGTTGTGTCAGG	Amplification of *VCX1* for complementation
VcxcompR	CACAACATCCAACCAAACACC	Amplification of *VCX1* for complementation
RTVcxF	ATCGCCTTGTTTGTCATTCCC	Amplification of *VCX1* for RT-PCR
RTVcxR	TCATCAGGACCATACCTTCCA	Amplification of *VCX1* for RT-PCR
RTACTF	CCTTCTACGTCTCTATCCAG	Amplification of *ACT1* for RT-PCR
RTACTR	TTTCAAGCTGAGAAGACTGG	Amplification of *ACT1* for RT-PCR
Vcx1FusF	CTGCACTGAGGAGCGCCCAATATCATA	Amplification of *VCX1* for Vcx1-mCherry fusion
Vcx1FusR	CTCGCCCTTGCTCACCATAGTTTTTTTT TTTTTTTTAGCCGGTGACG	Amplification of *VCX1* for Vcx1-mCherry fusion
cherryFusF	CGTCACCGGCTAAAAAAAAAAAAAAAA CTATGGTGAGCAAGGGCGAG	Amplification of *mCherry* for Vcx1-mCherry fusion
cherryFusR	CCAAGCTTGGTACCGAGCTC	Amplification of *mCherry* for Vcx1-mCherry fusion
Cch1RTF	GCAAACTCCGTCCCAACTAC	Amplification of *CCH1* for qRT-PCR

Cch1RTR	GCACCTCGTTCAGCAGATTC	Amplification of *CCH1* for qRT-PCR
Eca1RTF	GAGAGGCTTATGGCGAAAAC	Amplification of *ECA1* for qRT-PCR
Eca1RTR	ATGGCGAGGATGAATGAGAC	Amplification of *ECA1* for qRT-PCR
Pmc1RTF	GCCAACCAATCTCTTCGTAC	Amplification of *PMC1* for qRT-PCR
Pmc1RTR	TCTACCGCCTCTCTTACACC	Amplification of *PMC1* for qRT-PCR
Pmr1RTF	CGGCTTTATCGTACTTATCGG	Amplification of *PMC1* for qRT-PCR
Pmr1RTR	CCTTGCGTTTTGACATTCTC	Amplification of *PMC1* for qRT-PCR

4. Capítulo 2

The GATA-type transcriptional activator Gat1 regulates nitrogen uptake and metabolism in the human pathogen *Cryptococcus neoformans*

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The GATA-type transcriptional activator Gat1 regulates nitrogen uptake and metabolism in the human pathogen *Cryptococcus neoformans*

Lívia Kmetzsch [a], Charley Christian Staats [a,b], Elisa Simon [a], Fernanda L. Fonseca [c], Débora L. Oliveira [c], Luna S. Joffe [c], Jéssica Rodrigues [c], Rogério F. Lourenço [d], Suely L. Gomes [d], Leonardo Nimrichter [c], Marcio L. Rodrigues [c], Augusto Schrank [a,b], Marilene Henning Vainstein [a,b,*]

[a] *Centro de Biotecnologia, Universidade Federal do Rio Grande do Sul, Av. Bento Gonçalves 9500, 43421, Caixa Postal 15005, Porto Alegre, RS 91501-970, Brazil*
[b] *Departamento de Biologia Molecular e Biotecnologia, Universidade Federal do Rio Grande do Sul, Brazil*
[c] *Laboratório de Estudos Integrados em Bioquímica Microbiana, Instituto de Microbiologia Professor Paulo de Góes, Universidade Federal do Rio de Janeiro, Avenida Carlos Chagas Filho 373, CCS, Bloco I. Rio de Janeiro, RJ, 21941-902, Brazil*
[d] *Departamento de Bioquímica, Instituto de Química, Universidade de São Paulo, Av. Professor Lineu Prestes, 748, 05508-900, São Paulo, SP, Brazil*

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ABSTRACT

Nitrogen uptake and metabolism are essential to microbial growth. Gat1 belongs to a conserved family of zinc finger containing transcriptional regulators known as GATA-factors. These factors activate the transcription of Nitrogen Catabolite Repression (NCR) sensitive genes when preferred nitrogen sources are absent or limiting. *Cryptococcus neoformans GAT1* is an ortholog to the *Aspergillus nidulans AreA* and *Candida albicans GAT1* genes. In an attempt to define the function of this transcriptional regulator in *C. neoformans*, we generated null mutants (*gat1Δ*) of this gene. The *gat1* mutant exhibited impaired growth on all amino acids tested as sole nitrogen sources, with the exception of arginine and proline. Furthermore, the *gat1* mutant did not display resistance to rapamycin, an immunosuppressant drug that transiently mimics a low-quality nitrogen source. Gat1 is not required for *C. neoformans* survival during macrophage infection or for virulence in a mouse model of cryptococcosis. Microarray analysis allowed the identification of target genes that are regulated by Gat1 in the presence of proline, a poor and non-repressing nitrogen source. Genes involved in ergosterol biosynthesis, iron uptake, cell wall organization and capsule biosynthesis, in addition to NCR-sensitive genes, are Gat1-regulated in *C. neoformans*.

1. Introduction

Pathogenic fungi have to adapt and survive in distinct nutritive environments and, in this context, nitrogen uptake and its metabolism are critical to fungal growth. *Nitrogen Catabolite Repression* (NCR) is a mechanism that controls the fungi selective utilization of optimal nitrogen sources in preference to poor ones (Marzluf, 1997). NCR-sensitive genes are repressed when preferred nitrogen sources are available. Under limitation of these sources, the expression of genes encoding permeases and catabolic enzymes, required to utilize poor nitrogen sources, is activated by specific GATA-factor family of transcription factors. Members of this family contain a zinc-finger domain and are conserved in fungi (Coffman and Cooper, 1997; Coffman et al., 1996; Magasanik and Kaiser, 2002; Marzluf, 1997). In *Saccharomyces cerevisiae*, two GATA-factors involved in activation of NCR-sensitive genes (Gat1 and Gln3) were described (Stanbrough et al., 1995). The GATA-type transcriptional activator Gat1 and Gln3 orthologs of *Candida albicans* regulate nitrogen metabolism and virulence during host-pathogen interactions (Liao et al., 2008; Limjindaporn et al., 2003). The knockout of *GAT1* in *C. albicans* results in reduced capacity to metabolize some secondary nitrogen sources, but dimorphism is not impaired (Limjindaporn et al., 2003). The ortholog of *GAT1* in *Aspergillus fumigatus* (*AreA*) also participates in nitrogen regulation and virulence, since null mutants for *AreA* show attenuated virulence in a murine model of pulmonary aspergillosis (Hensel et al., 1998).

Exposure of *S. cerevisiae* cells to rapamycin mimics a low-quality nitrogen source, which results in activation of NCR-sensitive genes by Gat1 and Gln3 transcription factors (Hardwick et al., 1999; Scherens et al., 2006). This immunosuppressant drug inhibits a conserved signaling cascade for cell proliferation regulated by target of rapamycin (TOR) in response to nutrient availability (Jiang and Broach, 1999; Thomas and Hall, 1997). *S. cerevisiae* Gln3 and Gat1 are phosphorylated in a Tor-dependent mechanism, resulting in interaction with the cytoplasmic protein Ure2. Upon rapamycin treatment, Gln3 and Gat1 are dephosphorylated,

* Corresponding author at: Centro de Biotecnologia, Universidade Federal do Rio Grande do Sul, Avenida Bento Gonçalves 9500, 43421, Setor 4, Porto Alegre, RS 91501-970, Brazil. Fax: +55 51 3308 7309.
E-mail address: mhv@cbiot.ufrgs.br (M.H. Vainstein).

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released and directed to the nucleus, leading to transcription of NCR-sensitive genes (Beck and Hall, 1999; Bertram et al., 2000). Null mutants of *GLN3* or *GAT1* in *C. albicans* were resistant to rapamycin, suggesting that the TOR signaling pathway acts through Gln3 and Gat1 in this pathogen (Liao et al., 2008).

The life cycle of the human pathogen *Cryptococcus neoformans* is influenced by nitrogen availability, since in response to nitrogen limitation this organism initiates monokaryotic fruiting or mating (Wickes et al., 1996). These two processes lead to the production of spores, potential infectious propagules that are pathogenic in mice (Giles et al., 2009; Lengeler et al., 2000; Wickes et al., 1996). Furthermore, the *C. neoformans* ammonium permease Amt2, which is activated in response to nitrogen starvation, is required to induce ammonium-responsive invasive growth and mating, indicating the relevance of nitrogen metabolism in important aspects of *C. neoformans* biology (Rutherford et al., 2008). Here we report the identification of the GATA-type transcriptional activator Gat1 in *C. neoformans*, which influences nitrogen uptake and controls the transcription of genes involved in NCR, ergosterol biosynthesis, iron uptake, cell wall organization and capsule biosynthesis.

2. Material and methods

2.1. Fungal strains, plasmids and media

C. neoformans H99 strain was the recipient for target gene deletion. *C. neoformans* strains were maintained on YPD medium (1% yeast extract, 2% peptone, 2% dextrose, and 1.5% agar). YPD plates amended with hygromycin (200 μg/ml) were used to select *C. neoformans gat1* mutant strains. YPD plates amended with nourseothricin (100 μg/ml) were used to select *C. neoformans gat1*::*GAT1* complemented strains. Plasmid pJAF15 (Fraser et al., 2003) was the source of a hygromycin resistance cassette and plasmid pAI4 (Idnurm et al., 2004) was the source of a nourseothricin resistance cassette.

2.2. In silico analysis of the C. neoformans GATA-type transcription factor GAT1 ortholog

The putative *C. neoformans GAT1* gene sequence was identified by a BLAST search of the *C. neoformans* strain H99 genomic database at the Broad Institute using *GAT1* sequence of *S. cerevisiae* [GenBank: NP_116632.1]. The amino acid sequences of Gat1 orthologs from *S. cerevisiae*, *C. albicans, A. nidulans, Ustilago maydis, Neurospora crassa* and *C. neoformans* were aligned using ClustalX2 (Larkin et al., 2007). Mega4 was utilized for phylogenetic analysis applying the Neighbor-Joining method and the tree architecture was inferred from 1000 bootstraps (Tamura et al., 2007). Pfam database (http://pfam.sanger.ac.uk/) was used to search for conserved domains in the Gat1 ortholog proteins.

2.3. Disruption and complementation of C. neoformans GAT1

The Delsgate methodology (Garcia-Pedrajas et al., 2008) was employed for disruption of *GAT1*. The hygromycin resistance cassette from pJAF15 was subcloned into the EcoRV site of pDONR201 (Gateway donor vector, Invitrogen) to construct pDONRHYG plasmid. The 5′ and 3′ *GAT1* flanks (827 and 857 bp, respectively) were PCR amplified, and gel purified using Illustra GFX PCR DNA and Gel Band Purification kit (GE Healthcare). Approximately 300 ng of pDONRHYG vector and 30 ng of each PCR product were utilized in the BP clonase reaction, according to manufacturer's instructions (Invitrogen). This reaction was transformed into *Escherichia coli* OmniMAX 2-T1. After confirmation of the correct deletion construct, the plasmid was linearized by I-SceI digestion prior to *C. neoformans* biolistic transformation (Toffaletti et al., 1993). Transformants were screened by colony PCR, and the deletion was confirmed by Southern blot analysis and semi-quantitative RT-PCR. For complementation, a 6.2 Kb genomic PCR fragment containing the wild-type *GAT1* gene was cloned into the SmaI site of the pAI4 plasmid. The resulting plasmid was used for transformation of the *gat1* mutant strain. Random genomic insertion of the complemented gene was confirmed by Southern blot analysis and semi-quantitative RT-PCR. The primers utilized in these constructions are listed in Table S1.

2.4. Phenotypic characterization assays

Nitrogen source utilization was assessed in YCB medium (Yeast Carbon Base, Difco). Wild type (WT), *gat1* mutant and complemented strains were pre-cultured in YPD medium at 30 °C for 18 h. The cells were collected by centrifugation, washed three times with sterile dH_2O, suspended in YCB at a cell density of 10^8 cells/ml and incubated at 30 °C for 12 h to deplete nitrogen. Cells suspensions (10^7 cells/ml) were serially 10-fold diluted and 3 μl from each dilution was spotted onto YCB agar supplemented with 2 mM of each amino acid, 100 mM of urea, or 37 mM of ammonium sulfate. Sensitivity to rapamycin was assessed on YPD agar medium supplemented with 100 ng/ml and 200 ng/ml of rapamycin. WT, *gat1* mutant and complemented cells were cultured overnight in YPD, washed and suspended to a density of 10^7 cells/ml. The cells were diluted and spotted as described above. As control, cells were grown in YPD agar only. The plates were incubated for 2 days at 30 °C and photographed. Capsule formation was examined by microscopy after incubation for 24 h at 30 °C in a minimal medium and prepared with India ink. Relative capsule sizes were defined as the distance between the cell wall and the capsule outer border by cell diameter. ImageJ software was utilized to determine capsule measurements of one hundred cells of each strain. The content of extracellular glucuronoxylomannan (GXM) in culture supernatants was determined by ELISA (Fonseca et al., 2009).

2.5. Thin Layer Chromatography

Analyses of amino acids in culture supernatants of WT and *gat1* mutant strains were assessed by Thin Layer Chromatography (TLC). Briefly, starter cultures of WT and *gat1* mutant cells were grown overnight in YPD at 37 °C with shaking. Cells were washed three times, suspended in YNB (with no ammonia nor amino acids and amended with 2% of glucose) and incubated at 37 °C for 12 h to deplete nitrogen. Then, 10^8 cells of each strain were inoculated in YNB supplemented with 2 mM proline or aspartic acid. After 20 h incubation at 37 °C, the cells were removed by centrifugation for 10 min at 10,000*g*. The supernatants were passed through a 0.45 μm pore filter, followed by an ultra-filtration in membrane disks (1 kDa pore size). Silica gel TLC plates were spotted with 5 μl of each supernatant, and the mobile phase utilized for chromatography was butanol: acetic acid: water (40:10:10). For amino acids visualization, the plate was dipped in a ninhydrin solution (0.2% in ethanol) for 10 s. After 30 min incubation at 60 °C, the TLC plates were photographed. These analyses were performed in biological and technical duplicates.

2.6. Macrophage infection assay

The susceptibility of fungal cells to the antifungal action of phagocytes was determined by counting colony forming units (CFU) after interaction of WT, *gat1* mutant and *gat1*::*GAT1* complemented strains with the murine macrophage-like cell line RAW 264.7. Prior to interaction, fungal cells were opsonized with mono-

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clonal antibody 18B7 (1 μg/ml), a gift from Dr. Arturo Casadevall (Albert Einstein College of Medicine, USA). Macrophages were seeded at a concentration of 10^5 cells/well in a 96-well cell culture plate, and incubated overnight at 37 °C in 5% CO_2 in Dulbecco's modified Eagle's medium (DMEM) supplemented with 5% heat-inactivated fetal bovine serum (FBS). Fungal cells (10^6) were inoculated in each well, and after 1 h the wells were washed to remove unattached, extracellular fungal cells. After 20 h of incubation, infected cultures were again washed and sterile ice-cold dH_2O was added to each well to promote macrophage lysis. Fungal viability was measured by plating the lysates on YPD for CFU determination after cultivation of the plates for 48 h at 30 °C. The assay was performed in triplicate sets for each strain. Student's *t* test was used to determine the statistical significance of differences in fungal survival.

2.7. Virulence assay

Virulence studies were conducted according to a previously described intranasal inhalation infection model in mice (Cox et al., 2000). Fungal cells were cultured in 50 ml of YPD medium at 30 °C overnight with shaking, washed twice and re-suspended in PBS. Groups of eight female BALB/c mice (approximately 5 weeks old) were infected with 10^7 yeast cells suspended in 50 μl PBS and monitored daily. Kaplan–Meier analysis of survival was performed using GraphPad Prism Software. Animal studies were approved by the Federal University of Rio Grande do Sul Ethics Committee.

2.8. Microarray analysis

For RNA extraction, starter cultures of WT and *gat1* mutant cells were grown overnight in YPD at 37 °C with shaking. Cells were washed three times and suspended in YNB with no ammonia nor amino acids and amended with 2% of glucose. This medium was supplemented with 10 mM proline as a nitrogen source and incubated for 3 h at 37 °C. Three independent sets of RNA samples from independent experiments were prepared using Trizol reagent (Invitrogen) according to the manufacturer's protocol. After DNase treatment, RNA preparations were purified using RNAeasy mini columns (Qiagen). The CyScribe First-Strand cDNA Labeling Kit (GE Life Sciences) was utilized for preparation and purification of Cy3-dUTP and Cy5-dUTP labeled cDNA probes during first-strand cDNA synthesis reactions, according to the manufacture's protocol. These fluorescent labeled cDNAs were synthesized from 25 μg total RNA from each strain tested. Cy5-labeled cDNA from WT was mixed with Cy3-labeled cDNA from the *gat1* mutant strain and the mixture was hybridized to *C. neoformans* microarray slides for 18 h at 42 °C in a humid hybridization chamber. Arrays were washed with SSC buffer (1×) with 0.2% (w/v) SDS for 10 min at room temperature, followed by two washes in SSC (0.1×) with 0.2% (w/v) SDS for 5 min and scanned on a GenePix 4000B Scanner (Molecular Devices). Image preprocessing and data quantification was done using GenePix Pro Software and the raw expression data was obtained. For further data analysis, the TIGR microarray software suite (http://www.tm4.org/), which includes Midas and MeV software, was used. Data sets were first processed by Midas using total-intensity and LOWESS normalizations, and standard-deviation regulation, so that for each gene a normalized expression value was attributed. Following, MeV Software was used to perform one sample Student's *t* test in order to identify genes with statistically significant changes. Fold changes (WT/*gat1Δ*) were derived from the mean expression levels from three independent experiments. The complete list of differentially expressed genes (2-fold up or down regulated) with *P* values of <0.05 is presented in Table S2. The *C. neoformans* arrays (version 2) were purchased from an academic consortium of Genome Sequencing Center at School of Medicine in Washington University in St. Louis (http://genomeold.wustl.edu/activity/ma/cneoformans/).

2.9. Quantitative real time RT-PCR analysis

Real-time PCR reactions were performed in an Applied Biosystems 7500 Real-Time PCR System. PCR thermal cycling conditions were an initial step at 95 °C for 5 min followed by 40 cycles at 95 °C for 15 s, 60 °C for 20 s and 72 °C for 20 s. Platinum SYBR green qPCR Supermix (Invitrogen) was used as reaction mix, supplemented with 5 pmol of each primer and 2 μl of the cDNA template in a final volume of 25 μl. All experiments were done in three independent cultures and each cDNA sample was analyzed in duplicate with each primer pair. Melting curve analysis was performed at the end of the reaction to confirm a single PCR product. Data was normalized to actin cDNAs amplified in each set of PCR experiments. Relative expression was determined by the $2^{-\Delta CT}$ method (Livak and Schmittgen, 2001). The primers utilized in these experiments are listed in Table S1.

3. Results

3.1. Identification of a GATA-type transcription factor Gat1 ortholog in *C. neoformans*

The *GAT1* gene [Broad Institute: CNAG_00193.2] was identified in the *C. neoformans* var. *grubii* H99 genomic database available at the Broad Institute (http://www.broadinstitute.org/annotation/genome/cryptococcus_neoformans/MultiHome.html), based on its similarity to *GAT1* from *S. cerevisiae*. The *C. neoformans GAT1* coding region is 4239 bp long, contains three introns and encodes a putative 1277-amino-acid protein. Members of the GATA-factor family of transcription factors have a GATA zinc-finger domain [Pfam: PF00320] (Marzluf, 1997) that is also present in the *C. neoformans* Gat1 ortholog. Furthermore, a phylogenetic analysis including Gat1 sequences from different fungi species was performed (Fig. 1A) and showed that *C. neoformans* Gat1 is most similar to Gat1 from *U. maydis* and least similar to Gat1 from *S. cerevisiae*. The domain architecture of the *C. neoformans* Gat1 was compared to the orthologs herein analyzed showing that all orthologs have the GATA zinc-finger domain [Pfam: PF00320] plus a domain of unknown function DUF1752 [Pfam: 08550] (Fig. 1B).

3.2. The gat1 mutant exhibited normal growth only when arginine and proline were used as sole nitrogen sources

In order to perform a functional analysis of Gat1 in *C. neoformans*, knockout and complemented strains were constructed. Deletion and complementation of *GAT1* were confirmed by Southern blot analysis and semi-quantitative RT-PCR, as shown in Fig. 2. To assess the role of *C. neoformans GAT1* in nitrogen uptake and metabolism, the ability of WT, *gat1* mutant and *gat1*::*GAT1* complemented strains to grow in distinct nitrogen sources was evaluated. WT and complemented strains grew well on all nitrogen sources tested, but slightly growth differences between these strains were observed. However, the *gat1* mutant had strongly impaired growth on isoleucine, leucine, alanine, asparagine, lysine, aspartic acid, methionine, cysteine, phenylalanine, glutamic acid, threonine, glutamine, tryptophan, glycine, valine, serine, tyrosine, histidine, urea or ammonium sulfate as nitrogen sources. The *gat1* mutant exhibited normal growth only when the nitrogen sources utilized were arginine and proline (Fig. 3A). These *gat1* mutant phenotypes can be due to a nitrogen metabolism defect or to an amino acid transport defect. To address this point, TLC analyses

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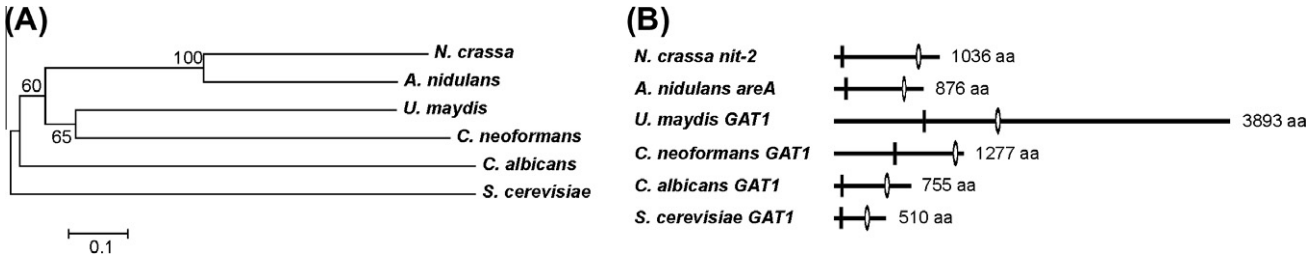

Fig. 1. Identification of a GATA-type transcriptional activator Gat1 ortholog in *C. neoformans* A. Phylogenetic analysis applying the Neighbor-Joining method including Gat1 sequences from distinct fungi, as follows: *S. cerevisiae* [Genbank: NP_116632.1], *C. albicans* [Genbank: AAP50501.1], *A. nidulans* [Genbank: XP_681936.1], *U. maydis* [Broad Institute: UM_04252], *N. crassa* [Genbank: AAB03891.1] and *C. neoformans* [Broad Institute: CNAG_00193.2]. The bar marker indicates the genetic distance, which is proportional to the number of amino acid substitutions. B. Domain architecture in the Gat1 orthologs of different fungi. The GATA zinc-finger domain [Pfam: PF00320] is represented by white ovals, and the domain of unknown function DUF1752 [Pfam: 08550] is represented by black bars. The length of each protein sequence (in amino acids) is indicated in the right.

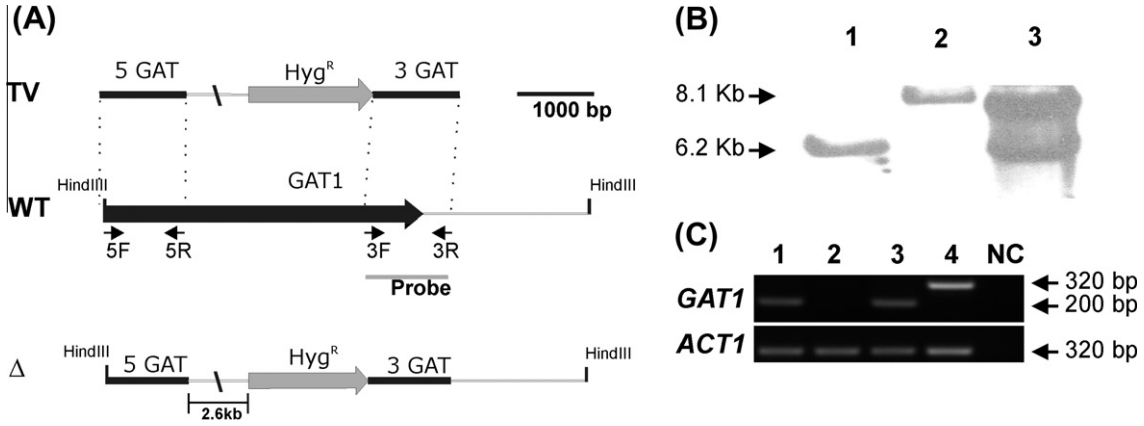

Fig. 2. Construction of *C. neoformans GAT1* knockout and complemented strains. A. Gat1 deletion scheme. TV represents the targeting vector constructed by Delsgate methodology. 5 GAT and 3 GAT represent the 5′ and 3′ gene flanks of *GAT1* gene, respectively. 5F and 5R: primers utilized to amplify 5′ flank of *GAT1* gene. 3F and 3R: primers utilized to amplify 3′ flank of *GAT1* gene. Hyg^R: cassette that confers hygromicin resistance. WT represents the wild type locus of the *GAT1* gene in H99 strain. *Δ* represents the *GAT1* locus in the *gat1* mutant strain. The cleavage sites of HindIII restriction enzyme are indicated. B. Confirmation by Southern blot. Genomic DNA (10 μg) from WT (lane 1), *gat1* mutant (lane 2) and *gat1*::*GAT1* complemented (lane 3) strains were digested with HindIII restriction enzyme. The 3′ gene flank was used as probe in Southern hybridization. Left numbers (in base pairs) indicate the hybridization signal sizes based upon the position of molecular size marker. C. Semi-quantitative RT-PCR using cDNA from WT (lane 1), *gat1* mutant (lane 2) and *gat1*::*GAT1* complemented (lane 3) strains as template. Right numbers (in bp) indicate the length of the transcript amplification for *GAT1* (upper panel) and *ACT1* (lower panel) genes. Lane 4: positive control using genomic DNA as template. NC: negative control of the PCR reaction.

of the supernatants of WT and *gat1* mutant cultures in the presence of proline or aspartic acid were performed. As seen in Fig. 3B, after 20 h of incubation, proline was completely utilized by both WT and *gat1* mutant strains, in contrast with aspartic acid, which was consumed only by WT strain. Since the aspartic acid concentration was not altered in *gat1* mutant supernatants in comparison to the concentration observed in the fresh medium, we can conclude that *gat1* mutant has a transport defect of some amino acids.

To test whether the TOR signaling pathway acts through the activity of *GAT1* in *C. neoformans*, as demonstrated for the orthologs in *C. albicans* and *S. cerevisiae* (Beck and Hall, 1999; Cardenas et al., 1999; Liao et al., 2008), we conducted rapamycin sensitivity assays. Unlike *C. albicans* and *S. cerevisiae*, the *C. neoformans gat1* mutant strain had almost identical sensibility to rapamycin in comparison to WT and complemented strains (Fig. 3C), indicating that the loss of *GAT1* does not influence the TOR signaling pathway in *C. neoformans*.

3.3. Disruption of GAT1 does not influence capsule size, but decreases extracellular GXM secretion

The *gat1* mutant strain was tested for its ability to grow at 37 °C, for capsule size and for melanin production since these traits are considered key virulence factors in *C. neoformans* (McClelland et al., 2005). The loss of *GAT1* apparently did not interfere with any of these traits (Fig. 4A and data not shown). The disruption of *GAT1*, however, decreased the levels of extracellular GXM, since lower polysaccharide contents were observed in culture supernatants from the *gat1* mutant strain, in comparison to WT ($P < 0.05$) (Fig. 4B). This finding suggests a role for *C. neoformans* Gat1 on the release of capsular polysaccharides to the extracellular environment.

3.4. Gat1 is neither required for C. neoformans survival during macrophage infection nor for virulence in a mouse intranasal model of cryptococcosis

The GATA-type transcriptional regulators Gat1 of *C. albicans* and AreA of *A. fumigatus* act in virulence of these human pathogens (Hensel et al., 1998; Limjindaporn et al., 2003). Therefore we tested whether *C. neoformans* Gat1 is essential during macrophage infection *in vitro* and in mice. Unlike as occurs in *C. albicans* and *A. fumigatus*, Gat1 does not influence *C. neoformans* virulence in mice (Fig. 4C). Furthermore, no differences were observed in survival of WT, *gat1* mutant and complement cells during an *in vitro* macrophage infection, as seen by CFU count showed in Fig. 4D.

3.5. The GATA-type transcriptional activator Gat1 regulates genes involved in NCR, ergosterol biosynthesis, iron uptake, cell wall organization and capsule biosynthesis in C. neoformans

Microarray analyses were conducted to identify target genes that are regulated by Gat1. We examined changes in global gene expression due to deletion of *GAT1* in the presence of proline, a

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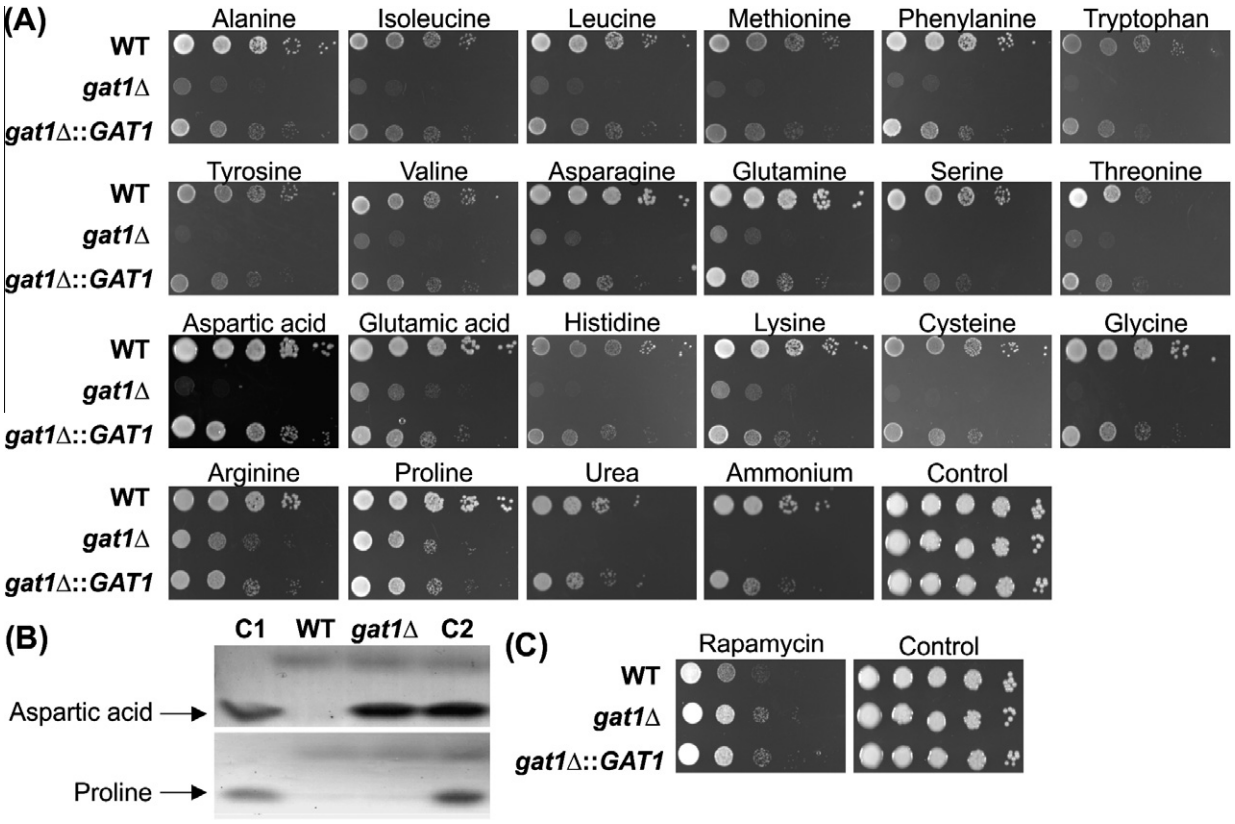

Fig. 3. Gat1 is involved in nitrogen uptake in *C. neoformans*. A. Nitrogen source utilization assay. Ten-fold serial dilutions of wild-type H99 (WT), *gat1* mutant (*gat1Δ*) and *gat1*::*GAT1* complemented (*gat1Δ*::*GAT1*) cells were plated in YCB medium supplemented with different nitrogen sources, as indicated. B. Thin Layer Chromatography. Culture supernatants of wild-type H99 (WT) and *gat1* mutant strains were analyzed for amino acids (proline or aspartic acid) visualization. As controls, 2 mM of each amino acid (C1) and fresh media supplemented with 2 mM of each amino acid (C2) were also spotted in the TLC plates. C. Rapamycin sensitivity assay. Ten-fold serial dilutions of wild-type H99 (WT), *gat1* mutant (*gat1Δ*) and *gat1*::*GAT1* complemented (*gat1Δ*::*GAT1*) cells were plated in YPD medium supplemented with 200 ng/ml of rapamycin. The plates were incubated for 2 days at 30 °C. As control, cells were grown in YPD agar only.

poor and non-repressing nitrogen source. We found that 127 genes were differentially expressed at least 2-fold in the WT strain. Among these, 54 genes were upregulated in WT in comparison to *gat1* mutant, including genes related to nitrogen metabolism and NCR mechanism (proline dehydrogenase, glutamate dehydrogenase, and amino acid transporter), iron uptake (high-affinity iron permease FTR1), and ergosterol biosynthesis (squalene monooxygenase). Seventy-three genes were found to be downregulated in the WT strain, including genes associated with capsule biosynthesis (capsular associated proteins), carbohydrate metabolism (alpha-L-rhamnosidase, UDP-glucose dehydrogenase, UDP-glucuronic acid decarboxylase, alpha 1–3 mannosyltransferase), cell wall integrity (chitin synthase 2), oxidative metabolism (copper zinc superoxide dismutase), and signal transduction (protein kinase C). Table 1 summarizes the microarray results. Some genes with less than 2-fold change were included in Table 1 due to their relevance to possibly explain the distinct phenotypes observed in the *gat1* mutant strain as that involved in capsule biosynthesis (capsular associated protein and alpha-1,3-mannosyltransferase) and nitrogen metabolism (glutamate-rich WD repeat containing one protein and amino acid transporter). The complete list of differentially expressed genes (2-fold up and down regulated) with *P* values of <0.05 is in Table S2. To validate the microarray results, the differential expression of seven selected genes was confirmed by quantitative real time RT-PCR (Fig. 5).

4. Discussion

Fungi can utilize a wide range of nitrogen sources and the key regulators that control nitrogen acquisition are well conserved in these organisms (Marzluf, 1997). In the present study, the GATA-type transcriptional activator Gat1 of *C. neoformans* was described. GATA-factors are responsible for activation of NCR-sensitive genes, as seen for Gat1 orthologs from *S. cerevisiae*, *C. albicans* and *A. fumigatus* (Hensel et al., 1998; Limjindaporn et al., 2003; Stanbrough et al., 1995). Members of the GATA-factor family of transcription factors are characterized by the presence of a GATA zinc-finger domain [Pfam: PF00320] (Marzluf, 1997). As expected, *C. neoformans* Gat1 is required for optimal growth on a variety of nitrogen sources, indicating that this GATA-factor is involved in nitrogen uptake and metabolism. A similar response was observed for *C. albicans* Gat1 and for Gln3, another GATA-type transcription factor that acts in parallel to Gat1 to control nitrogen uptake in this pathogen (Liao et al., 2008; Limjindaporn et al., 2003). Furthermore, *N. crassa nit-2* mutants had diminished growth on a diversity of amino acids sources including branched chain amino acids, tryptophan and phenylalanine, but not tyrosine (Facklam and Marzluf, 1978).

The TOR signaling pathway, that regulates cell proliferation in response to nutrient availability, is the target of inhibition by the drug rapamycin in *C. neoformans* and *C. albicans* (Cruz et al., 2001). Rapamycin binds to FKBP12 protein, and this complex interacts and inhibits the Tor1 kinase (Cruz et al., 2001). The sensitivity of *C. albicans* to rapamycin was greatly decreased by deletion of either *GLN3* or *GAT1* (Liao et al., 2008). However, *C. neoformans gat1* mutant strain had almost identical sensitivity to rapamycin in comparison to WT and complemented strains, indicating that the TOR signaling pathway does not act through Gat1 in *C. neoformans*. This phenotype may indicate the presence of redundant pathways or overlapping functions with other proteins that could

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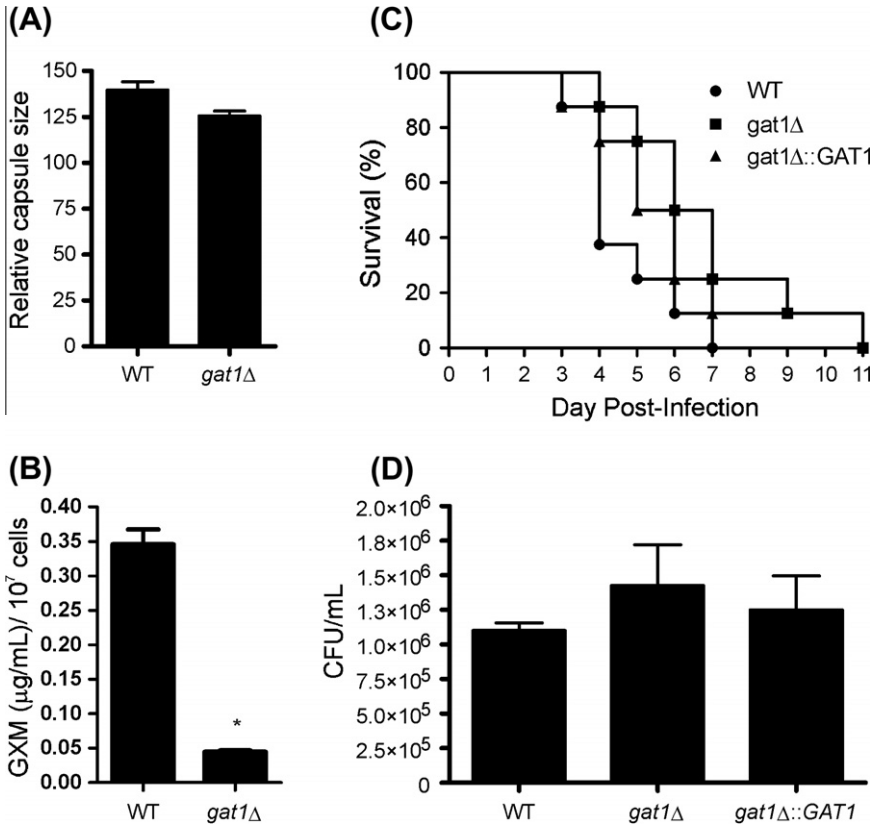

Fig. 4. Evaluation of virulence-related phenotypes of the *gat1* mutant strain. A. Relative capsule size of WT and *gat1* mutant cells. B. Content of extracellular GXM in culture supernatants of WT and *gat1* mutant cells determined by ELISA. *$P < 0.001$. C. Virulence assay of WT, *gat1* mutant and *gat1*::*GAT1* complemented strains in an intranasal inhalation infection model using BALB/c mice. D. Macrophage infection assay. CFU counting after macrophage infection with WT, *gat1* mutant and *gat1*::*GAT1* complemented strains.

Table 1
List of *GAT1*-regulated genes in *C. neoformans*.

Category	Description[a]/accession number at Broad Institute* or Genbank** databases	Fold change[b] (WT/gat1Δ)
Capsule biosynthesis	Capsular associated protein|CNK01140**	0.54
	Capsular associated protein|CNAG_00721*	*0.63*
Carbohydrate metabolism	Alpha-L-rhamnosidase|CNAG_02587*	0.50
	UDP-glucose dehydrogenase|CNAG_04969*	0.22
	UDP-glucuronic acid decarboxylase|CNG02560*	0.35
	Alpha-1,3-mannosyltransferase|CNAG_05142*	*1.54*
Cell wall integrity	Chitin synthase 2|CNAG_03326*	0.56
Ergosterol biosynthesis	Squalene monooxygenase|CNAG_06829*	2.02
	Squalene monooxygenase, putative|CND06110*	2.01
Iron metabolism	High-affinity iron permease CaFTR1|CNAG_02959*	2.45
Nitrogen metabolism	Proline dehydrogenase|CNAG_02049*	5.10
	Glutamate dehydrogenase (NADP+)|CNC00920**	11.37
	Glutamate-rich WD repeat containing 1|CNAG_01600*	*1.55*
	Amino acid transporter|CNAG_02539*	*1.42*
Oxidative metabolism	Copper zinc superoxide dismutase|CND01490**	0.22
Signal transduction	Protein kinase C, putative|CNC03300**	0.42
Transporters	Potassium transport protein|CNAG_02856*	0.33
	ATP-binding cassette (ABC) transporter|CND00300**	0.39
	Low-affinity zinc ion transporter, putative|CND00350**	0.45

[a] Descriptions were obtained from NCBI database (http://www.ncbi.nlm.nih.gov/) and Broad Institute Database (http://www.broadinstitute.org/annotation/genome/cryptococcus_neoformans/MultiHome.html).

[b] Data are presented as the average changes in expression of genes in WT cells compared with their expression in *gat1* mutant cells during growth in YNB supplemented with 10 mM proline as secondary nitrogen source. List contains a set of statistically significant genes identified by Student's *t* test that have average changes of greater than 2-fold. Genes with less than 2-fold change (italicized) are included in Table 1 due to their relevance to explain the distinct phenotypes observed in the *gat1* mutant strain.

compensate the loss of *GAT1* in *C. neoformans* during rapamycin treatment.

The deletion of *C. neoformans GAT1* did not affect capsule size, but decreased the levels of extracellular GXM in culture superna-

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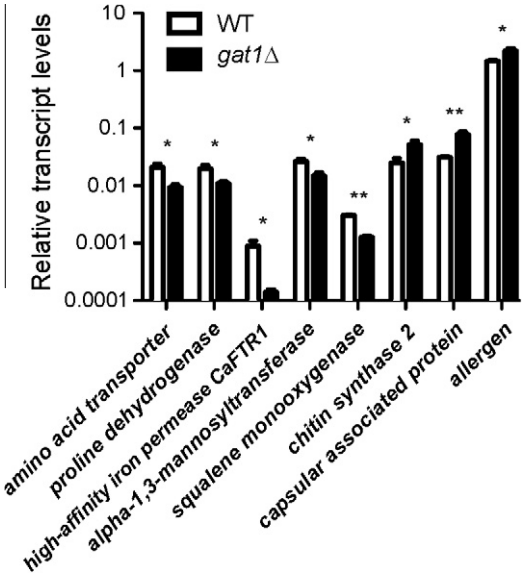

Fig. 5. Validation of the microarray results by quantitative real time RT-PCR. Relative expression of amino acid transporter, proline dehydrogenase, high-affinity iron permease FTR1, alpha-1,3-mannosyltransferase, squalene monooxygenase, chitin synthase 2, and capsular associated protein transcripts during growth of WT and *gat1* mutant cells in YNB amended with 10 mM of proline as nitrogen source. The measured quantity of the mRNA in each of the samples was normalized using the *Ct* values obtained for the actin gene. Data are shown as mean ± SD. $^{*}P < 0.05$. $^{**}P < 0.01$.

tants. Although the concentration of extracellular GXM is frequently associated with capsule enlargement, this same phenotype was observed for a *sec6* mutant of *C. neoformans* (Panepinto et al., 2009). We speculate that the basal levels of GXM secretion manifested by the *gat1* mutant are sufficient to warrant capsule assembly, although they are below the regular levels of polysaccharide secretion by *C. neoformans*. The facts that Gat1 orthologs of *C. albicans* and *A. fumigatus* are involved in virulence (Hensel et al., 1998; Limjindaporn et al., 2003), together with our observation of decreased GXM levels in *C. neoformans*, led us to test the role of Gat1 during macrophage infection *in vitro* and in a mice model of cryptococcosis. No differences between WT, *gat1* mutant and complemented strains were observed in these assays, indicating that *C. neoformans* Gat1 is not necessary for survival during host infection. We hypothesize that this observation may be related to the fact that arginine and proline are probably available in host tissues and fluids in their free form or obtained from the peptidase-mediated hydrolysis of host proteins (Pinti et al., 2007). For example, the utilization of serum amino acids by *C. albicans gat1* mutant and WT strains was examined. After 24 h of incubation, approximately 200 μM of proline (corresponding to 100%) had been consumed by both WT and *gat1* mutant strains, which indicate the availability and utilization of this amino acid by *C. albicans* in serum (Limjindaporn et al., 2003). Another possibility is that nitrogen transport in the *C. neoformans gat1* mutant in the *in vivo* environment differs from that in the *in vitro* environment, which could be sufficient for virulence and survival in macrophages. However, it remains unknown whether the observed phenotype for the *gat1* mutant *in vitro* also occurs during infection. Although the expression of *GAT1* was not required for fungal pathogenesis, we cannot rule out the possibility that its product and related proteins are required for infection, since nitrogen metabolism is relevant in essential aspects of *C. neoformans* biology (Kingsbury and McCusker, 2008; Kingsbury et al., 2004; Wickes et al., 1996).

C. neoformans transcription factor Gat1 activates the expression of known NCR-sensitive genes, as occurs with GATA-factors orthologs in other fungi. We found that genes encoding proline dehydrogenase, glutamate dehydrogenase, and amino acid transporter, the corresponding orthologs of *S. cerevisiae* PUT1, GDH2 and GAP1, respectively, are upregulated in the WT strain in comparison with *gat1* mutant during growth with proline as nitrogen source. The upregulation of such genes is in agreement with the observed effect on *S. cerevisiae* NCR-sensitive genes (Hofman-Bang, 1999; Scherens et al., 2006). *C. albicans* Gat1 also regulates the expression of the general amino acid transporter GAP1 ortholog (Limjindaporn et al., 2003).

The expression of genes related to capsule biosynthesis and carbohydrate metabolism are also controlled by *C. neoformans* Gat1 (Table 1). One of the genes encoding capsular associated protein is downregulated in the WT strain. Interestingly, UDP-glucose dehydrogenase and UDP-glucuronic acid decarboxylase genes are also downregulated. These enzymes directly participate in GXM biosynthesis, which may explain the reduced levels of extracellular GXM produced by the *gat1* mutant. The basic building units of GXM are UDP-glucuronic acid, UDP-xylose and GDP-mannose, and the connections between these units are done by glycosyltransferases (reviewed in Zaragoza et al. (2009)). Mannose is the most abundant sugar unit in GXM followed by glucuronic acid. The addition of glucuronic acid to the polysaccharide chain requires UDP-glucuronic acid synthesis, which occurs via oxidation of UDP-glucose by UDP-glucose dehydrogenase in the cytoplasm. Xylose is the third component of GXM. UDP-xylose, the target of fungal glycosyltransferases, is synthesized from the decarboxylation of UDP-glucuronic acid by UDP-glucuronic acid decarboxylase (*UGD1*) (reviewed in Zaragoza et al. (2009)). *C. neoformans UGD1* is essential for growth at 37 °C and for capsule biosynthesis (Moyrand and Janbon, 2004). The gene encoding a α-1,3-mannosyltransferase, which catalyzes the transfer of mannose from GDP-mannose to α-1,3-linked mannose disaccharides (Sommer et al., 2003), is upregulated in the WT in comparison to *gat1* mutant strain. Cryptococcal mannosyltransferase-1 gene (*CMT1*) was shown to be homologous to *C. neoformans CAP59*, a gene involved in capsule synthesis and GXM export (Garcia-Rivera et al., 2004; Zaragoza et al., 2009). The loss of *C. neoformans GAT1* negatively influences the contents of released GXM in culture supernatants. This phenotype, found in *gat1* mutant strain, can be related to the remodeled pattern of gene expression of key genes that act in the GXM biosynthesis. The expression of squalene monooxygenase, a rate-limiting step in sterol biosynthesis, is also reduced in the *gat1* mutant. A decrease in sterol production could potentially impair vesicle biogenesis and secretion through unconventional pathways, a mechanism used by *C. neoformans* to transport GXM to extracellular environments (Rodrigues et al., 2007).

The Sre1 transcription factor of *C. neoformans* regulates oxygen sensing, sterol homeostasis and virulence (Chang et al., 2007). Gene expression analysis of WT and *sre1* mutant strains in the presence of cobalt chloride (a hypoxia mimicking agent) revealed that genes involved in ergosterol biosynthesis and in iron/copper transport are Sre1-regulated (Lee et al., 2007). Surprisingly, at least 23 genes related to nitrogen metabolism were also regulated by Sre1 during growth with 0.6 mM $CoCl_2$, including *C. neoformans* transcription factor *GAT1* (transcriptional activator) [GenBank: CNA01820] (Lee et al., 2007). As seen for Sre1, Gat1 also regulates genes involved in ergosterol biosynthesis, cell wall integrity and iron uptake. These findings suggest that these two transcription factors are somehow linked and can act in parallel regulating important pathways in *C. neoformans.*

5. Conclusions

In conclusion, we have shown that Gat1, a GATA-type transcription factor in *C. neoformans*, is directly involved in nitrogen uptake

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and metabolism. Rapamycin sensitivity assays revealed that TOR signaling pathway does not act through Gat1 in *C. neoformans*. Additionally, Gat1 is neither required for *C. neoformans* survival during macrophage infection nor for virulence in a mouse intranasal model of cryptococcosis. As revealed by microarray analysis, Gat1 controls the transcription of NCR-sensitive genes, and genes involved in ergosterol biosynthesis, iron uptake, cell wall organization and capsule biosynthesis in *C. neoformans*.

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Appendix A. Supplementary material

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.fgb.2010.07.011.

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Table S1. **List of primers used in this study.**

Primer name	Sequence (5'-3')	Purpose
CnGAT5F	AAAATAGGGATAACAGGGTAATGACAAGCT TCCATGGCGCACT	Disruption construct for *GAT1*, 5' flank
CnGAT5R	GGGGACAAGTTTGTACAAAAAAGCAGGCT ATTGATCATGAGAGCTAGTCTGCT	Disruption construct for *GAT1*, 5' flank
CnGAT3F	GGGGACCACTTTGTACAAGAAAGCTGGGT ATAATAGTACTCCTAATCTTGC	Disruption construct for *GAT1*, 3' flank
CnGAT3R	AAAAATTACCCTGTTATCCCTAACCCGGAG GCTTTAAGAATCC	Disruption construct for *GAT1*, 3' flank
GATcompF	TTTGTTGGTGGTTGTGTCAGG	Amplification of *GAT1* for complementation
GATcompR	TCATTTACCGCAGCCCCTTCC	Amplification of *GAT1* for complementation
RTGATF	TCTATCATGCCTACTGGTGAG	Amplification of *GAT1* for RT-PCR
RTGATR	GATGAAGCGACACTGTTCTTG	Amplification of *GAT1* for RT-PCR
RTACTF	CCTTCTACGTCTCTATCCAG	Amplification of *ACT1* for RT-PCR
RTACTR	TTTCAAGCTGAGAAGACTGG	Amplification of *ACT1* for RT-PCR
RTPROF	TTGGACGCCTACACTCTTCTC	Amplification of proline dehydrogenase transcript for qRT-PCR
RTPROR	TGGGTTGGTTGACGACATAG	Amplification of proline dehydrogenase transcript for qRT-PCR
RTAATF	CAACAACGCAGGCATCAAG	Amplification of amino acid transporter transcript for qRT-PCR
RTAATR	CCAACGCAAGAGCATACAGG	Amplification of amino acid transporter transcript for qRT-PCR
RTFTRF	GGCGGTAACAAGGAAGATG	Amplification of *FTR1* transcript for qRT-PCR

RTFTRR	TGATGAAAGGCAGGAAGAAG	Amplification of *FTR1* transcript for qRT-PCR
RTMANF	TCGCACTGGGAGTCAAGATG	Amplification of alpha-1,3-mannosyltransferase transcript for qRT-PCR
RTMANR	AATGGAGCAGGGAGAAGGAAC	Amplification of alpha-1,3-mannosyltransferase transcript for qRT-PCR
RTSQUF	ATGAAAAGGGGGAAGTGAG	Amplification of squalene monooxigenase transcript for qRT-PCR
RTSQUR	AGGAGAGGGGATTGAAGTG	Amplification of squalene monooxigenase transcript for qRT-PCR
RTCHS2F	TACCTCCTCCTTCTTCCTTC	Amplification of chitin synthase 2 transcript for qRT-PCR
RTCHS2R	CTTCTACATCCTCTTGCTTC	Amplification of chitin synthase 2 transcript for qRT-PCR
RTCAPF	GGCGTGGTAGTGGTGTGAAC	Amplification of capsular associated protein transcript for qRT-PCR
RTCAPR	CGGAGAGGGTGCTGATGAG	Amplification of capsular associated protein transcript for qRT-PCR

Table S2. Complete list of Gat1-regulated genes with differential expression of at least 2-fold.

Accession number/Description[a]	Fold change[b]	Gene mean[c]	Gene SD[d]	P value	Array A	Array B	Array C
CNA01460 |expressed protein	0.454169396	-1.1386976	0.082300656	0.03250741	ND	-1.0805023	-1.1968929
CNA02100 | multidrug resistance protein, putative	0.495397983	-1.0133401	0.293918	0.026915714	-1.1394007	-1.2232004	-0.6774192
CNA02900 | hypothetical protein	0.409591931	-1.2877408	0.32692194	0.020815307	-1.1909915	-1.6521182	-1.0201127
CNA02900 | hypothetical protein	0.451574774	-1.1469632	0.030739704	2.3934434E-4	-1.1824157	-1.1307453	-1.1277286
CNA05260 | conserved hypothetical protein	0.489001501	-1.0320892	0.0755337	0.0017805931	-1.0454439	-1.1000549	-0.9507688
CNA05650 | endopeptidase, putative	0.476787858	-1.0685806	0.11271197	0.0036880479	-1.1064789	-0.94180393	-1.15745897
CNA06340| conserved hypothetical protein	0.396375144	-1.3350616	0.28946194	0.015310699	-1.66822	-1.1917696	-1.1451952
CNA06340 | conserved hypothetical protein	0.40918467	-1.289176	0.2926515	0.016747048	-1.5492148	-1.3460517	-0.9722615
CNA06950 | electron carrier, putative	0.479816508	-1.0594453	0.19912483	0.011571327	-1.1644739	-1.1840677	-0.8297943
CNA07260 |succinate-CoA ligase (ADP-forming), putative	0.298269094	-1.7453136	0.42088258	0.018838437	-1.7005556	-1.3485986	-2.1867866
CNA07260 | succinate-CoA ligase (ADP-forming), putative	0.299724433	-1.7382914	0.5518076	0.03198699	-1.6496797	-1.2361518	-2.3290427
CNAG_00002 | predicted protein	0.484342263	-1.0459012	0.18478021	0.0102445865	-0.8501084	-1.0703633	-1.2172319
CNAG_00456 | conserved hypothetical protein	0.406893715	-1.2972761	0.48528197	0.043615706	-1.4484318	-0.7544055	-1.688991

CNAG_00456 | conserved hypothetical protein	0.409995648	-1.2863195	0.41640168	0.0332008	-1.4029146	-0.8240485	-1.6319954
CNAG_00581 | endopeptidase	0.430827008	-1.2148194	0.11551908	0.0030005756	-1.3309032	-1.0998734	-1.2136816
CNAG_00654 | conserved hypothetical protein	0.447438747	-1.1602379	0.34788424	0.028684586	-1.5058182	-1.1648009	-0.8100946
CNAG_00790 | PAP2 domain-containing protein	0.505273772	-0.9848628	0.022246456	0.010167479	-1.0005934	-0.9691322	-0.9848628
CNAG_00794 | conserved hypothetical protein	0.501539502	-0.99556476	0.07652544	0.034568008	ND	-0.9414531	-1.04967642
CNAG_01314 | vacuolar protein	0.46885535	-1.0927852	0.3872459	0.03940108	-0.93936443	-1.5332345	-0.80575667
CNAG_01345 | mitochondrial ribosomal protein subunit S2	0.492841005	-1.0208058	0.24659741	0.018902445	-1.2891843	-0.96901804	-0.80421506
CNAG_01355 | thiol-disulfide exchange intermediate	0.486817623	-1.0385467	0.17317024	0.009140848	-1.0789468	-0.8487476	-1.1879457
CNAG_01735 | conserved hypothetical protein	0.459517465	-1.1218084	0.099291146	0.0026011495	-1.2096423	-1.0140742	-1.1417087
CNAG_01735 | conserved hypothetical protein	0.496399167	-1.0104274	0.402823	0.04910842	-1.2069703	-0.54706055	-1.27725135
CNAG_02587 | alfa-L-rhamnosidase	0.502422647	-0.9930266	0.054214165	0.024564132	ND	-0.9546914	-1.0313618
CNAG_02822 | conserved hypothetical protein	0.476254094	-1.0701966	0.39255595	0.042041175	-1.522656	-0.867644	-0.8202898
CNAG_02856 | potassium transport protein	0.326209454	-1.6161295	0.13578968	0.002344932	-1.6363355	-1.4713691	-1.7406839

CNAG_02856 | potassium transport protein	0.335988579	-1.5735159	0.0389365	2.0404096E-4	-1.5378218	-1.6150378	-1.5676881
CNAG_03221 | ribosomal protein L29	0.452667771	-1.1434755	0.37587884	0.034182042	-0.7491986	-1.1834702	-1.4977577
CNAG_03221 | ribosomal protein L29	0.500308944	-0.99910885	0.2108488	0.014522906	-1.07929	-1.1581049	-0.75993165
CNAG_03250 | conserved hypothetical protein	0.451287305	-1.1478819	0.14848012	0.0055310316	-0.9970523	-1.2938954	-1.152698
CNAG_03439 | conserved hypothetical protein	0.493942599	-1.0175847	0.36038473	0.039357338	-1.0158032	-0.658094	-1.3788569
CNAG_03507 | mitochondrial processing peptidase beta subunit	0.344217202	-1.5386089	0.39388427	0.02115465	-1.4021777	-1.98257	-1.231079
CNAG_03507 | mitochondrial processing peptidase beta subunit	0.354879243	-1.4945999	0.21313602	0.0067104837	-1.3363881	-1.7369655	-1.4104461
CNAG_03566 | conserved hypothetical protein	0.383240624	-1.3836776	0.43726593	0.03171388	-1.8169085	-1.391639	-0.9424853
CNAG_04869 | carboxylesterases	0.443347548	-1.17349	0.15047553	0.005436245	-1.1340097	-1.0466906	-1.3397697
CNAG_04969 | UDP-glucose dehydrogenase	0.216057992	-2.2105095	0.14532927	0.0014376817	-2.3529484	-2.216127	-2.0624531
CNAG_04969 | UDP-glucose dehydrogenase	0.238365244	-2.0687542	0.018875888	0.004107311	ND	-2.0821013	-2.0554071
CNAG_05159 | hypothetical	0.474584537	-1.075263	0.23667651	0.015768552	-1.0213338	-1.3342502	-0.870205

protein							
CNAG_06347 | conserved hypothetical protein	0.48881481	-1.0326401	0.09275932	0.0026788479	-1.0519103	-0.9317592	-1.1142508
CNAG_06402 | conserved hypothetical protein	0.36102151	-1.4698433	0.48112503	0.033909004	-2.0053072	-1.0738925	-1.3303302
CNAG_06567 | conserved hypothetical protein	0.468255388	-1.0946325	0.36262017	0.034688048	-1.4247588	-1.1526295	-0.7065092
CNAG_06669 | conserved hypothetical protein	0.4400085	-1.1843967	0.17769551	0.007419639	-1.3670934	-1.1739318	-1.0121649
CNAG_06669 | conserved hypothetical protein	0.492878754	-1.0206953	0.13084236	0.005432913	-0.8732368	-1.1229151	-1.065934
CNAT_00207_CNAG_00207_cneo	0.454318639	-1.1382236	0.2777438	0.019275783	-1.2931442	-1.3039533	-0.8175733
CNAT_01838_CNAG_01838_cneo	0.42096827	-1.2482166	0.009255785	0.0033379854	-1.2547615	-1.2416718	-1.2482165
CNAT_07107_CNAG_07107_cneo	0.482923475	-1.0501335	0.1964692	0.011467219	-1.050778	-1.2462796	-0.8533429
CNB00910 | expressed protein	0.501479593	-0.9957371	0.13174792	0.005784897	-0.8499814	-1.0308794	-1.1063505
CNB02230 | conserved hypothetical protein	0.496335689	-1.0106119	0.2724312	0.023376731	-0.85647714	-0.8501912	-1.32516736
CNB03630 | hypothetical protein	0.458717833	-1.1243211	0.2324829	0.013954515	-1.334768	-0.8747634	-1.1634319
CNB04190 | mitochondrial import receptor subunit tom40, putative	0.455879677	-1.133275	0.33781752	0.028364962	-1.5128578	-0.8656481	-1.0213191
CNBC3050_CNBC3050 gene_cneo_B-3501[a]	0.497609178	-1.006915	0.3169939	0.03148467	-1.1012088	-1.2660632	-0.653473
CNBC3050_CNBC3050 gene_cneo_B-3501[a]	0.498182656	-1.0052533	0.26970732	0.023164118	-1.0735791	-1.2342267	-0.7079541

CNBC3800_CNBC3800 gene_cneo_B-3501[a]	0.47953938	-1.0602788	0.14539866	0.0062101083	-0.91674227	-1.2074707	-1.05662343
CNBD5570_CNBD5570 gene_cneo_B-3501[a]	0.469602795	-1.0904871	0.13724773	0.0052387156	-1.1681414	-1.1713023	-0.9320176
CNBD5570_CNBD5570 gene_cneo_B-3501[a]	0.483970863	-1.0470079	0.12800471	0.004945382	-1.1350924	-1.1057569	-0.9001744
CNBD5880_CNBD5880 gene_cneo_B-3501[a]	0.376045073	-1.4110225	0.19020213	0.0060023004	-1.4455076	-1.5816228	-1.2059371
CNBD5880_CNBD5880 gene_cneo_B-3501[a]	0.40706427	-1.2966715	0.2070916	0.00839552	-1.4327934	-1.3988752	-1.0583459
CNBK3440_CNBK3440 gene_cneo_B-3501[a]	0.440183874	-1.1838218	0.30793577	0.021818705	-0.94786036	-1.5321633	-1.07144174
CNC00700 | PRCDNA38, putative	0.456428252	-1.13154	0.17770118	0.008120899	-1.2669183	-0.9303134	-1.1973883
CNC01350 | expressed protein	0.402136725	-1.314242	0.45674896	0.0379819	-0.96133685	-1.151264	-1.83012515
CNC03210 | Unknown	0.456948506	-1.1298965	0.16746302	0.0072427075	-1.2443836	-0.9376959	-1.20761
CNC03300 | protein kinase C, putative	0.426802638	-1.228359	0.2546517	0.014025205	-1.255558	-1.4683194	-0.9611996
CNC03420_CNC03420 gene_cneo_JEC21	0.462973428	-1.1109987	0.23019648	0.014010276	-1.1921797	-0.85121065	-1.28960575
CNC03420_CNC03420 gene_cneo_JEC21	0.49602643	-1.0115111	0.26129666	0.021527922	-1.167831	-0.70985836	-1.15684394
CND00300 | ATP-binding cassette (ABC) transporter, putative	0.398393606	-1.3277336	0.44698784	0.035764404	-1.7971172	-0.9071456	-1.278938
CND00300 | ATP-binding cassette	0.398469471	-1.3274589	0.21797605	0.008868431	-1.4342533	-1.4714439	-1.0766795

(ABC) transporter, putative							
CND00350 | low-affinity zinc ion transporter, putative	0.455270284	-1.1352048	0.20616232	0.01081579	-1.1653003	-1.3246652	-0.9156489
CND01490 | copper zinc superoxide dismutase	0.194513744	-2.362056	0.3624362	0.00775685	-2.2021875	-2.1070402	-2.7769403
CND01490 | copper zinc superoxide dismutase	0.243107364	-2.0403345	0.18867652	0.002838309	-1.9847218	-1.8857147	-2.250567
CND01910 | conserved hypothetical protein	0.47642836	-1.0696688	0.22992955	0.015054811	-0.8073148	-1.2361361	-1.1655555
CND01910 | conserved hypothetical protein	0.503091165	-0.99110824	0.14924487	0.0074738655	-0.8208242	-1.0533037	-1.09919682
CND04320 | chaperone, putative	0.449482484	-1.1536632	0.2250901	0.012452652	-1.2306337	-0.90018445	-1.33017145
CND05690 | MFalpha3	0.358721221	-1.479065	0.21347435	0.006872278	-1.6299123	-1.572476	-1.2348067
CND05690 | MFalpha3	0.475582752	-1.0722317	0.3414281	0.03217632	-1.0958288	-1.4012492	-0.7196171
CND05750 | pheromone alpha	0.31462214	-1.6683079	0.06470578	5.010557E-4	-1.6459204	-1.7412345	-1.6177688
CND05750 | pheromone alpha;	0.33825182	-1.5638304	0.14512756	0.0028584725	-1.410769	-1.6994472	-1.581275
CNE00740 | translation initiation factor, putative	0.4969005	-1.0089711	0.1775678	0.010166864	-1.1205473	-0.8042085	-1.1021575
CNE02480 | ATP binding protein, putative	0.488353757	-1.0340015	0.11895922	0.0043829856	-1.1223358	-1.0809337	-0.898735
CNF03780 | malate dehydrogenase, putative	0.343268933	-1.5425888	0.08456697	0.0010002947	-1.4560148	-1.5467575	-1.6249941
CNF03840 | 40s ribosomal protein s15, putative	0.456005996	-1.1328753	0.343612	0.029323434	-0.76489687	-1.1883612	-1.44536783

CNG00370 | Unknown	0.460276535	-1.1194272	0.043223195	0.017377138	ND	-1.1499907	-1.0888637
CNG00860 | mitochondrial processing peptidase beta subunit, mitochondrial precursor (beta-mpp)	0.314429721	-1.6691905	0.29829794	0.010478504	-1.6743715	-1.3683358	-1.9648642
CNG00860 | mitochondrial processing peptidase beta subunit, mitochondrial precursor (beta-mpp), putative	0.367671766	-1.4435097	0.34821647	0.018850425	-1.3101965	-1.1816461	-1.8386865
CNG01990 | g1/s-specific cyclin pcl1 (cyclin hcs26), putative	0.437250246	-1.1934689	0.43766257	0.042021263	-1.1198659	-0.7972746	-1.6632662
CNG02560 | UDP-glucuronic acid decarboxylase Uxs1p	0.350372561	-1.5130383	0.35442883	0.017803963	-1.6194758	-1.117587	-1.8020521
CNI00360 | oxidoreductase, putative	0.414197026	-1.2716109	0.072711065	0.025726158	ND	-1.3230253	-1.2201965
CNI01400 | choline kinase, putative	0.409209601	-1.2890881	0.19424261	0.0074835224	-1.212543	-1.5099417	-1.1447796
CNK00130 | thioredoxin peroxidase, putative	0.281002993	-1.8313426	0.019679451	3.8489336E-5	-1.8207439	-1.819234	-1.8540499
CNL05770 | conserved hypothetical protein	0.420877094	-1.2485291	0.14518249	0.0044769864	-1.4125016	-1.1363316	-1.1967541
CNL05770;conserved hypothetical protein;	0.499024101	-1.0028186	0.32346177	0.032974325	-1.3203963	-0.6737775	-1.014282
CNL05950 | hypothetical protein	0.351169779	-1.5097594	0.58458394	0.04651616	-1.5073371	-2.0955508	-0.9263903

CNL05950 | hypothetical protein	0.413024967	-1.2756991	0.2645997	0.014039112	-1.3587699	-1.4887959	-0.9795315
CNM00840 | expressed protein	0.5034712	-0.99001884	0.39675465	0.049586594	-1.3042754	-0.54419285	-1.12158827
CNM02040 | conserved hypothetical protein	0.508995759	-0.97427446	0.13473395	0.006314535	-0.8238282	-1.0151805	-1.08381468
CNN00760 | hypothetical protein	0.504410586	-0.98732954	0.3096507	0.031257644	-0.8028649	-0.8143014	-1.34482232
CNA00430 | conserved hypothetical protein	3.003891908	1.5868329	0.15836392	0.044850864	1.4748527	ND	1.6988131
CNA02690 | conserved hypothetical protein	2.156945464	1.1089897	0.17729387	0.008412094	1.1490378	1.2628341	0.9150972
CNA02730 | expressed protein	2.11704348	1.0820509	0.4300035	0.048818707	0.60871416	1.4485931	1.18884544
CNA04550 | defender against cell death 1 (dad-1), putative	2.15071506	1.1048164	0.25130123	0.016812285	1.0996056	0.85616124	1.35868236
CNAG_00070 | conserved hypothetical protein	2.093508245	1.0659226	0.27312785	0.021192417	1.3019575	1.129053	0.7667573
CNAG_00405 | STE/STE20/YSK protein kinase	2.099859789	1.070293	0.24217689	0.016641457	0.8883319	1.3451682	0.9773789
CNAG_01044 | ceramidase	2.063804194	1.0453061	0.34073403	0.03364086	0.7717736	0.9371548	1.4269899
CNAG_01072 | amidase	2.042345012	1.0302266	0.098705925	0.0030458742	0.92775995	1.1246837	1.03823615
CNAG_01493 | conserved hypothetical protein	2.104786354	1.0736738	0.3950489	0.042285223	0.68509537	1.0610366	1.47488943
CNAG_02017 | predicted protein	2.161043631	1.1117282	0.27512926	0.019810632	1.4219106	0.8971705	1.0161035
CNAG_02017 | predicted protein	2.356239681	1.2364863	0.34940934	0.02559992	1.6227665	0.94246215	1.14423025
CNAG_02046 | poly(A)-binding protein binding protein	2.733771383	1.4508926	0.2079537	0.006778108	1.3919705	1.2787578	1.6819495

CNAG_02046 | poly(A)-binding protein binding protein	2.759915315	1.464624	0.17091782	0.00450875	1.3615584	1.3703967	1.6619169
CNAG_02049 | proline dehydrogenase	4.920385665	2.2987714	0.15011258	0.0014183932	2.3206396	2.4367504	2.1389242
CNAG_02049 | proline dehydrogenase	5.495049587	2.4581325	0.24974477	0.0034231548	2.5122998	2.6763484	2.1857493
CNAG_02089 | conserved hypothetical protein	2.104956471	1.0737904	0.27953836	0.02185257	1.382895	0.8387243	0.9997519
CNAG_02089 | conserved hypothetical protein	2.258188158	1.1751657	0.3462324	0.027736342	1.5574855	1.085251	0.8827606
CNAG_02864 | predicted protein	3.631862288	1.8607095	0.42369407	0.016847748	1.3918276	1.9741997	2.2161012
CNAG_02864 | predicted protein	3.65066984	1.8681612	0.38980874	0.014204385	1.4273077	2.0099301	2.1672458
CNAG_02959 | high-affinity iron permease CaFTR1	2.40830915	1.2680206	0.09835793	0.001999589	1.2545309	1.3724272	1.1771037
CNAG_02959 | high-affinity iron permease CaFTR1 (2178 nt)	2.509887848	1.3276229	0.07413127	0.0010376617	1.3685621	1.3722565	1.2420501
CNAG_02969 | thioesterase family protein	2.064523299	1.0458087	0.22898176	0.015606858	0.9331679	0.89496577	1.30929243
CNAG_03424 | conserved hypothetical protein	2.208950209	1.1433609	0.18070504	0.008223742	1.1192386	0.9759285	1.3349156
CNAG_03494 | conserved hypothetical protein	2.464528902	1.3013119	0.13079867	0.0033507005	1.1656258	1.3117068	1.4266031
CNAG_03494 | conserved hypothetical protein	2.927034803	1.5494399	0.14968327	0.0030963852	1.3952482	1.5589063	1.6941652

CNAG_03995 | predicted protein	2.012798985	1.0092031	0.1831008	0.010795062	1.1522382	0.80284625	1.07252485
CNAG_03995 | predicted protein	2.04476876	1.0319377	0.20318969	0.012678107	1.2150918	0.8133686	1.0673527
CNAG_03996 | guanosine-diphosphatase	2.391001773	1.2576152	0.3319371	0.022442952	1.6128243	1.2047186	0.9553027
CNAG_04034 | conserved hypothetical protein	2.157080624	1.1090801	0.20122483	0.010795404	0.9828452	1.0032597	1.3411354
CNAG_04760 | cytoplasmic protein	3.178910282	1.6685323	0.35161355	0.014481918	1.6331466	2.0365007	1.3359496
CNAG_04760 | cytoplasmic protein	3.305408549	1.7248286	0.4240364	0.019557143	1.6911244	2.1647112	1.3186502
CNAG_05346 | peroxisomal sarcosine oxidase	2.639222349	1.4001129	0.09107133	0.029260233	1.46451	ND	1.3357158
CNAG_05397 | conserved hypothetical protein	2.269498917	1.1823738	0.12298518	0.046739258	1.2693374	ND	1.0954102
CNAG_05515 | conserved hypothetical protein	2.174428092	1.120636	0.29085332	0.021725152	1.4374563	1.0587368	0.8657149
CNAG_06237 | YeeE/YedE family protein	2.523427609	1.3353847	0.34843627	0.021949654	1.5641375	0.9343692	1.5076474
CNAG_06297 | conserved hypothetical protein	2.523144095	1.3352226	0.15606414	0.004522966	1.4757866	1.3625997	1.1672815
CNAG_06297 | conserved hypothetical protein	2.582005726	1.3684922	0.510468	0.04338411	1.0438936	1.9568835	1.1046995
CNAG_06446 | mitochondrial splicing suppressor	2.332761153	1.2220386	0.25902706	0.014647873	1.0110277	1.1439661	1.511122

CNAG_06829 | squalene monooxygenase	2.022699215	1.0162818	0.1344844	0.005786446	1.039056	1.1379251	0.8718643
CNAT_00945_CNAG_00945_cneo	2.941154762	1.5563827	0.2210307	0.0066557713	1.8114027	1.420031	1.4377144
CNAT_02098_CNAG_02098_cneo	2.32610029	1.2179133	0.36347783	0.028429437	1.5314935	0.8195311	1.3027153
CNB05100 | hypothetical protein	2.075190241	1.0532436	0.3200532	0.029427858	0.72945666	1.3694276	1.06084654
CNB05590 | hypothetical protein	2.877954134	1.5250436	0.39052832	0.021166908	1.7021616	1.7956285	1.0773407
CNB05590 | hypothetical protein	2.913304095	1.5426563	0.44372636	0.026487678	1.8212099	1.7758027	1.0309563
CNBB0690_CNBB0690 gene_cneo_B-3501[a]	3.202776665	1.6793232	0.061935723	0.016598683	1.6355281	ND	1.7231183
CNBG1310_CNBG1310 gene_cneo_B-3501[a]	2.026718893	1.019146	0.15503043	0.0076251836	1.1981157	0.9262269	0.9330954
CNBG1310_CNBG1310 gene_cneo_B-3501[a]	2.061076852	1.0433983	0.059354186	0.0010769096	1.0861083	1.0684631	0.9756235
CNBJ3230_CNBJ3230 gene_cneo_B-3501[a]	3.385652021	1.7594337	0.2602121	0.0072122314	1.849723	1.4661033	1.9624748
CNBJ3230_CNBJ3230 gene_cneo_B-3501[a]	3.56376449	1.833402	0.18531804	0.0033883406	1.8184817	1.6559951	2.0257292
CNBK3360_CNBK3360 gene_cneo_B-3501[a]	2.529763644	1.3390026	0.34635827	0.02158374	0.9527168	1.6218759	1.4424151
CNBK3360_CNBK3360 gene_cneo_B-3501[a]	2.565549391	1.3592678	0.29281273	0.01511861	1.0231165	1.5588237	1.4958632
CNBN1140_CNBN1140 gene_cneo_B-3501[a]	3.093249703	1.6291233	0.4226064	0.021703152	1.4361299	2.1137712	1.3374688
CNBN1140_CNBN1140	3.402546552	1.7666149	0.64670587	0.041882932	1.55225	2.4932845	1.2543102

gene_cneo_B-3501							
CNC00160 | phosphopyruvate hydratase, putative	2.053835165	1.0383204	0.3752903	0.040893674	1.0622064	1.4010973	0.6516575
CNC00160 | phosphopyruvate hydratase, putative	2.206164396	1.1415403	0.40930432	0.040282138	1.1106662	1.5654074	0.7485473
CNC00730 | Unknown	3.624256844	1.8576852	0.17687143	0.0030080627	1.6621448	2.0065088	1.904402
CNC00920 | glutamate dehydrogenase (NADP+), putative	11.29626552	3.497774	0.312611	0.0026520002	3.155731	3.7686985	3.5688925
CNC00920 | glutamate dehydrogenase (NADP+), putative	11.45570943	3.5179949	0.3012289	0.0024349673	3.1883588	3.778958	3.5866679
CNC01320_CNC01320 gene_cneo_JEC21	2.986828495	1.5786144	0.5460785	0.0376493	1.2371787	1.2902374	2.2084271
CNC01320_CNC01320 gene_cneo_JEC21	2.990615631	1.5804425	0.42573753	0.023344625	1.4613364	1.2269428	2.0530483
CND06110 | squalene monooxygenase, putative	2.015579517	1.0111947	0.125882	0.0051261	0.9165816	1.1540656	0.9629369
CNF03350 | hypothetical protein	3.723007442	1.8964685	0.2088368	0.0040177046	1.7141618	1.8509252	2.1243185
CNI02240 | conserved hypothetical protein	2.201064771	1.1382016	0.087482	0.001963348	1.1270639	1.0568217	1.2307192
CNI02240 | conserved hypothetical protein	2.468146106	1.3034278	0.13024086	0.0033115994	1.2075826	1.2509863	1.4517145
CNI03850 | expressed protein	2.046854426	1.0334085	0.19364536	0.01150283	1.0307221	1.2283831	0.8411203
CNI04150 | cytosine-purine permease, putative	2.117771007	1.0825466	0.029697044	2.5075444E-4	1.1126708	1.0816729	1.0532961

CNI04150 | cytosine-purine permease, putative	2.161201967	1.1118339	0.09351546	0.002349811	1.2186258	1.0722854	1.0445905
CNJ01900 | cytoplasm protein, putative	3.4185279	1.7733752	0.59780383	0.03585393	1.3292663	2.4530811	1.5377782
CNJ01900 | cytoplasm protein, putative	3.480001433	1.7990879	0.46248585	0.021325683	1.8336798	2.2433066	1.3202773
CNK00070 | phosphoketolase, putative	2.35969942	1.2386031	0.49366	0.049084637	1.3506343	1.6666194	0.6985556
CNK00070 | phosphoketolase, putative	2.462020032	1.2998425	0.31154242	0.01861536	1.3184896	1.6016426	0.9793953
CNK01410 | hypothetical protein	2.224852664	1.1537098	0.27705196	0.018685345	0.84803116	1.3882694	1.22482884
CNK01410 | hypothetical protein	2.493159185	1.317975	0.18371694	0.0064145816	1.1763194	1.5255587	1.2520469
CNM01400 | conserved hypothetical protein	2.61283967	1.3856186	0.23337853	0.009324076	1.1379373	1.6014159	1.4175026
CNM01400 | conserved hypothetical protein	2.735362809	1.4517322	0.18436931	0.0053333226	1.2463174	1.6028711	1.5060081
CNN01000 | hypothetical protein	2.116024307	1.0813562	0.41273722	0.04528774	0.81743985	0.8696384	1.55699035
CNN01000 | hypothetical protein	2.338441277	1.2255472	0.16806525	0.0062103225	1.055834	1.2288929	1.3919147
CNN01150 | expressed protein	2.51869582	1.3326769	0.4137566	0.030660562	0.89638644	1.7194415	1.38220276
CNN01150 | expressed protein	2.801594196	1.486248	0.4431777	0.028382456	1.0180885	1.8992871	1.5413684
CNN01210 | hypothetical protein	2.151628196	1.1054288	0.34634468	0.031198377	1.3379538	1.2709532	0.7073794

a. Descriptions were obtained from NCBI database (http://www.ncbi.nlm.nih.gov/) and Broad Institute Database (http://www.broadinstitute.org/annotation/genome/cryptococcus_neoformans/MultiHome.html).

b. Data are presented as the average changes in expression of genes in WT cells compared with their expression in *gat1* mutant cells during growth in YNB supplemented with 10mM proline as secondary nitrogen source. List contains all statistically significant genes identified by Student's *t* test ($P < 0.05$) that have average changes greater than 2-fold.

c. Mean of expression levels of three independent experiments: Array A, B and C.

d. Standard deviations of Gene means.

5. Capítulo 3

A role for Golgi reassembly and stacking protein in virulence and polysaccharide secretion in *Cryptococcus neoformans*

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Role for Golgi reassembly and stacking protein in virulence and polysaccharide secretion in *Cryptococcus neoformans*

Lívia Kmetzsch[1§], Luna S. Joffe[3§], Charley C. Staats[1], Débora L. de Oliveira[3], Radames J. Cordero[4], Arturo Casadevall[4,5], Leonardo Nimrichter[3], Augusto Schrank[1,2], Marilene H. Vainstein[1,2†], and Marcio L. Rodrigues[3†*].

[1] Centro de Biotecnologia and [2] Departamento de Biologia Molecular e Biotecnologia, Universidade Federal do Rio Grande do Sul, Av. Bento Gonçalves 9500, 43421, Caixa Postal 15005, Porto Alegre, RS 91501-970, Brazil; [3]Laboratório de Estudos Integrados em Bioquímica Microbiana, Instituto de Microbiologia Professor Paulo de Góes, Universidade Federal do Rio de Janeiro, 21941-902, Brazil; [4]Department of Microbiology and Immunology and [5]Division of Infectious Diseases of the Department of Medicine, Albert Einstein College of Medicine, 1300 Morris Park Ave, Bronx, NY, 10461, USA.

[†] MHV and MLR share senior authorship on this article.

[§] LK and LSJ contributed equally to this work.

(*) Corresponding author. Mailing address: Avenida Carlos Chagas Filho 373, CCS, Bloco I. Instituto de Microbiologia, UFRJ. Rio de Janeiro, RJ, Brasil, 21941-902. Phone 55 21 2598 3035; Fax 55 21 2560 8344. Email: marcio@micro.ufrj.br.

Abstract

Secretion of virulence factors to the extracellular milieu is a critical pathogenic mechanism for the establishment of cryptococcosis, a disease caused by the yeast pathogen *Cryptococcus neoformans*. One key virulence strategy of *C. neoformans* is the release of glucuronoxylomannan (GXM), a wall-associated immune-modulatory polysaccharide that reaches the extracellular space through secretory vesicles. Golgi reassembly and stacking protein (GRASP) is required for unconventional protein secretion mechanisms in different eukaryotic cells, but its role in polysaccharide secretion is unknown. In this study, we demonstrate that a *C. neoformans* mutant lacking expression of a GRASP ortholog displayed attenuated virulence in an animal model of cryptococcosis, in comparison to wild type (WT) and reconstituted cells. Mutant cells formed defective surface-associated polysaccharide capsules and showed a reduced ability to secrete GXM. Isolation of GXM from cultures of WT, reconstituted or mutant strains followed by effective diameter determination by light scattering revealed that yeast cells lacking expression of the GRASP ortholog produced polysaccharides with reduced dimensions. Production of extracellular vesicles by *grasp* mutant cells was comparable to that observed for wild type and reconstituted strains. Vesicular concentration of GXM, however, was significantly smaller on fractions obtained from cultures of the *grasp* mutant. These results demonstrate that GRASP, a protein involved in unconventional protein secretion, is also required for polysaccharide secretion and contributes to the virulence in *C. neoformans*.

Introduction

Cryptococcus neoformans is a yeast-like pathogen associated with high mortality rates in immunosuppressed individuals (Park et al., 2009; Prado et al., 2009). *C. neoformans* virulence is dependent on the expression of a number of virulence factors, including enzymes, pigments, polysaccharides and lipids (Li and Mody, 2010). Like many bacterial pathogens, *C. neoformans* is surrounded by a polysaccharide capsule (Zaragoza et al., 2009). Capsule formation in this fungal pathogen requires intracellular polysaccharide synthesis (Feldmesser et al., 2001; Yoneda and Doering, 2006), followed by secretion of capsular components to the extracellular space and their incorporation into the cell surface (Rodrigues et al., 2008b; Zaragoza et al., 2009). Capsule expression is purportedly the most important constraint for cryptococcal virulence (McClelland et al., 2005).

The major capsular component of *C. neoformans*, glucuronoxylomannan (GXM), is presumably synthesized in the Golgi and targeted to the cell surface (Yoneda and Doering, 2006). The polysaccharide then traverses the cell wall in vesicles that reach the extracellular space (Rodrigues et al., 2007). In this milieu, GXM is used for enlargement of the cryptococcal capsule (Zaragoza et al., 2006). It has been demonstrated that the exposure of *C. neoformans* to brefeldin A, which affects the formation of Golgi-related transport vesicles, results in a strong inhibition of capsule assembly (Hu et al., 2007). Consequently, the Golgi apparatus seems to be required for GXM synthesis and secretion, based on results of two independent studies. Yoneda and Doering demonstrated that a *C. neoformans* mutant lacking expression of

Sav1p, a putative secretory vesicle-associated Rab GTPase that is essential for exocytosis, accumulates intracellular, post-Golgi vesicles containing GXM (Yoneda and Doering, 2006). These findings are in agreement with those described by Panepinto and colleagues, who showed that *C. neoformans* cells with deficient expression of Sec6p, which mediates polarized targeting of secretory vesicles to active sites of exocytosis, had a decreased rate of GXM secretion (Panepinto et al., 2009). Although both studies clearly indicated an association of Golgi-derived pathways with GXM secretion, the fact that capsular expression was apparently normal in both *sav1* and *sec6* mutants suggested that other components of Golgi-associated secretory pathways could have a role in GXM traffic in *C. neoformans*.

Golgi reassembly and stacking proteins (GRASPs) have been implicated in the stacking of Golgi cisternae, vesicle tethering, and mitotic progression (Nickel and Seedorf, 2008; Nickel and Rabouille, 2009). GRASP is primarily attached peripherally to the cytoplasmic surface of Golgi membranes, but its distribution into other cellular compartments is also expected (Nickel and Rabouille, 2009). In *Dictyostelium discoideum*, the single GRASP orthologue (GrpA) is required for unconventional secretion of acyl-coenzyme A-binding protein (AcbA) during spore differentiation (Kinseth et al., 2007), in a process that requires secretory vesicles (Cabral et al., 2010). GRASP is also required for the delivery of integrin alpha subunits to the plasma membrane of *Drosophila melanogaster* in a Golgi-independent manner (Schotman et al., 2008). More recently, it has been demonstrated that starvation-induced-

secretion of AcbA in *Saccharomyces cerevisiae* and *Pichia pastoris* also requires GRASP (Duran et al., 2010; Manjithaya et al., 2010).

The fact that many of the cryptococcal virulence factors are exocellular components (Li and Mody, 2010) implies that secretory activity is directly linked to virulence in *C. neoformans*. Many of the *C. neoformans* virulence factors are released to the extracellular space through apparently unconventional mechanisms of secretion (Rodrigues et al., 2007; Nosanchuk et al., 2008; Rodrigues et al., 2008a; Rodrigues et al., 2008b; Casadevall et al., 2009). The apparent link between cryptococcal virulence and unconventional secretion led us to investigate the role of GRASP in an animal model of *C. neoformans* infection. Our results suggest that virulence is attenuated in a *grasp* mutant of *C. neoformans*, which is associated to a clear defect in the ability of yeast cells to secrete GXM. To our knowledge, this is the first report showing a role for GRASP in microbial virulence and in polysaccharide secretion in eukaryotic cells.

Material and methods

Fungal strains, plasmids and media. *C. neoformans* H99 strain was employed as a recipient for creating target gene deletion. Plasmid pJAF15, which contains the hygromycin marker cassette, was a generous gift of Joseph Heitman (Duke University, Durham, NC USA). Plasmid pAI4, which contains the nourseothricin marker cassette, was kindly provided by Alexander Idnurm (University of Missouri-Kansas City Kansas City, MO USA). The strains were maintained on YPD medium (1% yeast extract, 2% peptone, 2 % dextrose, and 1,5% agar). YPD plates containing hygromycin (200 µg/ml) were used to select *C. neoformans grasp* deletion transformants (*grasp* strain). YPD plates containing nourseothricin (100 µg/ml) were used to select *C. neoformans grasp* reconstituted transformants (*grasp::GRASP* strain).

***In silico* analysis of the *C. neoformans GRASP* ortholog.** The putative *C. neoformans GRASP* gene sequence was identified by a BLAST search of the *C. neoformans* var. *grubii* strain H99 genomic database at the Broad Institute using *GRASP* sequences of *S. cerevisiae* (GenBank accession number NP_593015) and *D. discoideum* (GenBank accession number EAL60823). The aminoacid sequences of *GRASP* orthologs from *Rattus norvegicus*, *Caenorhabditis elegans*, *Drosophila melanogaster*, *Saccharomyces cerevisiae*, *Dictyostelium discoideum*, *Schizosaccharomyces pombe*, *Plasmodium falciparum*, *Aspergillus nidulans*, *Ustilago maydis*, *Candida albicans* and *C. neoformans* were aligned using ClustalX2 (Larkin et al., 2007). Mega4 were utilized for phylogenetic analysis applying the Neighbor-Joining method and the tree architecture was inferred from 10,000 bootstraps (Tamura et al., 2007). The

identification of the PDZ domains in the sequences was performed by BLAST search of the previous sequences with the *R. norvegicus* PDZ domains as previously described (Kinseth et al., 2007).

Disruption and complementation of GRASP. Disruption of GRASP was achieved employing the Delsgate methodology (Garcia-Pedrajas et al., 2008). A Gateway cloning system donor vector (Invitrogen) containing the hygromycin selectable marker for *C. neoformans* transformation was constructed. A 2.2 Kb PCR product spanning the hygromycin marker cassette fragment was amplified from pJAF15 and cloned into the EcoRV site of pDONR201 (Invitrogen). The resulting vector was named pDONRHYG. The 5' and 3' GRASP flanks (702 bp and 700 bp, respectively) were PCR amplified and purified from agarose gels (Illustra GFX PCR DNA and Gel Band Purification kit, GE Healthcare). Approximately 300 ng of pDONRHYG and 30 ng of each PCR product were submitted to BP clonase reaction, according to manufacturer's instructions (Invitrogen). This reaction was transformed into *Escherichia coli* OmniMAX 2-T1. After confirmation of the correct deletion construct, the plasmid was linearized with I-SceI prior to *C. neoformans* biolistic transformation (Toffaletti et al., 1993). The mutants were screened by colony PCR, and the deletion was confirmed by Southern blot and semi-quantitative RT-PCR analyses. For complementation, a 3 Kb genomic PCR fragment containing the wild-type *GRASP* gene was cloned into the SmaI site of pAI4. The resulting plasmid was used for transformation of the *grasp* mutant strain. Genomic insertion of the complemented gene was confirmed by Southern blot and semi-quantitative RT-PCR analyses. The primers utilized in these plasmid constructions are listed in

Table 1. The strategy used for generation of *C. neoformans grasp* mutant strain is summarized in Figure 1.

Virulence assay. Virulence studies were conducted according to a previously described intranasal inhalation infection model (Cox et al., 2000) using eight female BALB/C mice (approximately 6 weeks old) for each strain. Mice were infected with 10^7 yeast cells suspended in 50 µl of saline and monitored daily. Animal studies were approved by the Federal University of Rio Grande do Sul Ethics Committee. Kaplan–Meier analysis of survival was performed using GraphPad Software.

Phagocytosis of *C. neoformans* cells by murine macrophages. Murine macrophage-like cells (RAW 264.7 lineage) were obtained from the American Type Culture Collection (ATCC). Cultures were maintained and grown to confluence in 25 cm^2 culture flasks containing Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% (vol/vol) fetal bovine serum (FBS) at 37 °C and 5% CO_2. The culture medium was replaced with fresh media for further incubation with fluorescein isothiocyanate (FITC)-labeled *C. neoformans* yeast cells. These cells were prepared by first staining yeast cells with 0.5 mg/ml FITC in PBS for 10 min. Suspensions of FITC-labeled *C. neoformans* were then prepared in DMEM to generate a ratio of 10 yeasts per host cell. Interactions between fungal and host cells occurred at 37 °C and 5 % CO_2 for 18 h. Cells were washed three times with PBS to remove non-adherent yeasts. Fungi-host cell complexes were then treated for 10 min at 25 ^oC with trypan blue (200 µg/ml) to discriminate between surface-associated and intracellular yeast cells. After removal from the plastic surface with a cell scrapper, the cells

were analyzed by flow cytometry. Control preparations were developed as described above using uninfected cells and non-stained yeast.

GXM and capsule determination. *C. neoformans* cells (WT, *grasp* mutant and reconstituted strains) were placed onto glass slides and mixed with similar volumes of India ink. The suspensions were covered with glass coverslips and analyzed with an Axioplan 2 (Zeiss, Germany) microscope. Images were acquired using a Color View SX digital camera and processed with the software system analySIS (Soft Image System). Capsule sizes, defined as the distances between the cell wall and the outer border of the capsule in India ink stained yeast cells, were determined by using the ImageJ Software (version 1.33), elaborated and provided by National Institutes of Health (NIH, http://rsb.info.nih.gov/ij/). Cell diameters were determined using the same software. Final measurements were presented as ratio of capsule size / cell diameter. Similar cellular suspensions were analyzed by fluorescence microscopy. The staining reagents used in fluorescence microscopy included calcofluor white, which has been extensively used to stain chitin in fungal cell walls, and the monoclonal antibody (mAb) 18B7, a mouse IgG1 with high affinity for GXM of different cryptococcal serotypes (Casadevall et al., 1998). Yeast cells (10^6) were suspended in 4% paraformaldehyde cacodylate buffer (0.1 M, pH 7.2) and incubated for 30 min at room temperature. Fixed yeast cells were washed twice in PBS and incubated in 1% bovine serum albumin in PBS (PBS-BSA) for 1 h. The cells were then suspended in 100 µl of a 25 µM calcofluor white solution (Invitrogen) for 30 min at 37^{o}C. After washing in PBS, the cells were incubated for 1 h in the presence of mAb 18B7 (1 µg/ml). The cells were

finally incubated with a fluorescein isothiocyanate (FITC) labeled goat anti-mouse IgG (Fc specific) antibody (Sigma). For negative control we used an isotype-matched irrelevant IgG at the same concentrations used for mAb 18B7. Cell suspensions were mounted over glass slides and analyzed under an Axioplan 2 (Zeiss, Germany) fluorescence microscope. Images were acquired and processed as described above.

Determination of GXM concentration in fungal supernatants. Culture supernatants were obtained as recently described (Fonseca et al., 2010). GXM concentration in fungal supernatants was determined by ELISA with mAb 18B7A1, using modifications of a previously described protocol for GXM detection (Casadevall et al., 1992; Fonseca et al., 2009). Briefly, 96-Well polystyrene plates were coated with standard GXM, supernatant samples for further blocking with bovine serum albumin. The plates were sequentially incubated with mAb 18B7 and an alkaline phosphatase-conjugated goat anti-mouse IgG1 for 1 h. Reactions were developed after the addition of *p*-nitrophenyl phosphate disodium hexahydrate, followed by measuring absorbance at 405 nm with a microplate reader (TP-reader, Thermo Plate). Antibody concentration in this assay corresponded to 1 μg/ml.

Analysis of virulence factors in the *grasp* mutant. Urease activity and melanin formation, two well defined *C. neoformans* virulence factors that are related to vesicular secretion (Rodrigues et al., 2008a; Eisenman et al., 2009), were used as prototypes in assays aiming at the evaluation of virulence determinants that are not associated to capsule expression. Urease activity was assayed after growth of WT, *grasp* and reconstituted strains in Christensen's

agar medium at 30°C for 48h (Cox et al., 2000). This medium contains 300 mM urea and phenol red as a pH indicator. The urease activity is expected to convert urea into ammonia, resulting in increase of the pH of the medium. This feature is reflected by a color change from yellow to bright pink. An *ure1* mutant was utilized as a negative control. Analysis of melanin production followed the methodology described by Baker and colleagues (Baker et al., 2007). WT, mutant and complemented cells were inoculated in a chemically defined medium containing L-asparagine (1g/L), $MgSO_4 \cdot 7H_2O$ (0.5 g/l), KH_2PO_4 (3g/l) and thiamine (1 mg/l), supplemented with 1 mM L-3,4-dihydroxyphenylalanine (L-DOPA). *C. neoformans* cells were then cultivated at 30°C. After 24 h intervals, the cultures were centrifuged 1,000 x *g* for 10 min; pellets were photographed for visual analysis of pigmentation and the supernatants were spectrophotometrically analyzed (absorbance 492 nm).

GXM effective diameter. For diameter determination, extracellular GXM was isolated from culture supernatants as previously described by our group (Nimrichter et al., 2007). Yeast cells were cultivated in a minimal medium composed of glucose (15 mM), $MgSO_4$ (10 mM), KH_2PO_4 (29.4 mM), glycine (13 mM), and thiamine-HCl (3 µM), pH 5.5, for two days at room temperature with shaking and separated from culture supernatants by centrifugation at 4,000 *g* (15 min, 4^oC). The supernatant fluids were collected and again centrifuged at 15,000 *g* (15 min, 4^oC), to remove smaller debris. The pellets were discarded and the resulting supernatant was concentrated approximately 20-fold using an Amicon (Millipore, Danvers, MA) ultrafiltration cell (Nimrichter et al., 2007). After supernatant concentration, the viscous layer formed was collected with a cell

scraper and transferred to graduated plastic tubes for GXM determinations. The effective diameter of GXM in these samples was determined by Quasi elastic light scattering in a 90Plus/BI-MAS Multi Angle Particle Sizing analyzer (Brookhaven Instruments Corp., Holtsville, NY), according with the method described by Frases and colleagues (Frases et al., 2009). Polysaccharide diameter was regulated by incubation of dialyzed fractions with 1 mM $CaCl_2$ for 1 h at room temperature.

Vesicle isolation and sterol determination. Extracellular vesicle fractions from cultures of WT, mutant or reconstituted *C. neoformans* strains at similar cell densities were isolated from culture supernatants, using variations of methods previously described (Rodrigues et al., 2007; Albuquerque et al., 2008; Rodrigues et al., 2008a). Yeast cells and debris were removed by sequential centrifugation at 4,000 and 15,000 *g* (15 min, 4^{o}C). Supernatants were collected and concentrated by approximately 20-fold using an Amicon ultrafiltration system (cutoff = 100 kDa). The concentrate was again centrifuged at 4,000 and 15,000 *g* (15 min, 4^{o}C) and passed through filtering membranes (0.8 μm pores). Filtered fractions were finally centrifuged at 100,000 *g* for 1 h at 4^{o}C. Pellets were then washed by three sequential suspension and centrifugation steps, each consisting of 100,000 *g* for 1 h at 4^{o}C with 0.1 M Tris buffered saline (TBS, pH 7.4). The resulting pellets were then suspended in methanol and then nine volumes of chloroform were added. The mixture was vigorously vortexed and centrifuged to discard precipitates, dried by vacuum centrifugation and partitioned according to (Folch et al., 1957). The lower phase, containing neutral lipids, was recovered for analysis by high performance thin layer

chromatography (HPTLC). The lipid extract was loaded into HPTLC silica plates (Si 60F254s, LiChrospher, Merck, Germany) and separated using a solvent system containing hexane:ether:acetic acid (80:40:2, v/v/v). The plate was sprayed with a solution of 50 mg ferric chloride ($FeCl_3$) in a mixture of 90 ml water, 5 ml acetic acid and 5 ml sulfuric acid. After heating at 100°C for 3-5 min, the sterol spots were identified by the appearance of a red-violet color. Stained HPTLC plates were digitalized using Adobe Photoshop CS (version 8.0) and densitometrically analyzed with the Scion Image software (version Alpha 4.0.3.2). The presence of GXM in vesicle fractions was determined by ELISA, as described previously by our group (Rodrigues et al., 2007).

Results

We identified a putative *GRASP* gene in the *C. neoformans* var. *grubii* (serotype A) genomic database (Broad Institute, accession number CNAG_03291.2). The *GRASP* coding region is 1051 basepairs long, contains four introns and encodes a putative 256-amino-acid protein. GRASP proteins are characterized by the presence of two PDZ-like domains in the N-terminal region (Kinseth et al., 2007). A BLAST search using the PDZ domains sequences from *R. norvegicus* GRASP revealed that these regions are also present in the *C. neoformans* GRASP ortholog. Sequence comparisons revealed that the identity range for the PDZ-1 and PDZ-2 domains were 40 and 48%, respectively (Figure 2A). Furthermore, a broad phylogenetic analysis including GRASP sequences from distinct eukaryotic organisms were performed. The phylogeny tree split into two major clades. The clade C-II contained the GRASPs from the majority of the fungi analyzed, all ascomicetes. The clade C-I harbored the GRASP from the basidiomicetous fungi *C. neoformans* and *U. maydis*, as well those from mammalian (*R. norvegicus*), fly (*D. melanogaster*), worm (*C. elegans*), protist (*P. falciparum*), and amoeba (*D. discoideum*) (Figure 2B).

The relevance of extracellular molecules in the cryptococcal pathogenesis (Li and Mody, 2010) and the recently described roles of GRASP in unconventional secretion (Kinseth et al., 2007; Levi and Glick, 2007; Schotman et al., 2008; Manjithaya et al., 2010; Duran et al., 2010) led us to evaluate whether the GRASP knockout would affect the virulence of *C. neoformans*. Intranasal infection with WT cells caused death of all animals in 12 days (Figure 3A). Similarly, all mice infected with the reconstituted

grasp::GRASP strain died 11 days post-infection. When animals were infected with the *grasp* mutant strain, however, 100% killing was observed only 18 days post-infection ($P<0.01$, in comparison to both WT and reconstituted cells). These results indicate that the *grasp* mutant had attenuated virulence in an animal model of cryptococcosis. Since interaction with macrophages is considered to be determinant in a number of fungal infections (Seider et al., 2010), we evaluated whether the attenuated virulence of the *grasp* mutant was related to decreased levels of association of *C. neoformans* with macrophages. Our results indicate that WT, *grasp* and complemented strains show similar levels of association with murine phagocytes (Figure 3B). Treatment of the macrophage-fungi complexes with trypan blue resulted in a decreased of fluorescence levels corresponding to 8, 12 and 7% for WT, *grasp* and reconstituted cells, respectively (data not shown). The relatively high resistance of infected macrophages to lose fluorescence after exposure to trypan blue indicates that, in all systems, *C. neoformans* cells were internalized by the macrophages in high rates.

Pigmentation, urease activity and synthesis and release of capsular components are associated with the survival of *C. neoformans* during infection of host cells (reviewed in (Gong et al., 2009; Zaragoza et al., 2009)). We therefore analyzed the ability of the *grasp* mutant to pigment, to produce extracellular urease activity, to secrete GXM and to form a polysaccharide capsule. Levels of pigmentation and urease activity in cultures of WT, *grasp* and reconstituted cells were similar (Figure 4). The *grasp* mutant, however, manifested a hypocapsular phenotype, in comparison to WT and reconstituted

cells (Figure 5). This observation was apparently related to a defective ability to secrete GXM, since the concentration of this major capsular component in mutant cell culture supernatants was significantly smaller than measured in supernatants of WT and reconstituted cultures. Fluorescence microscopy revealed that GXM was detected at the cell surface of both WT and reconstituted cells, as well as in the *grasp* mutant. In the latter, some of the cells appeared to lack a detectable capsule, while other cells showed the hypocapsular phenotype observed by India ink counterstaining. These results suggest that the reduced amounts of extracellular GXM in *grasp* mutant supernatants reflect a decrease in polysaccharide concentration rather than an altered reactivity with the antibody to GXM. The regular pattern of calcofluor white staining observed for all strains indicated that the *GRASP* knockout did not affect the architecture of the cell wall.

The suggestion that disruption of *GRASP* affected GXM secretion led us to analyze the presence of GXM in secretory extracellular vesicles, which have been demonstrated to be responsible for GXM export in *C. neoformans* (Rodrigues et al., 2007). Sterols are structural components of fungal extracellular vesicles and are markers of vesicle membranes (Rodrigues et al., 2007; Oliveira et al., 2010). For this reason, these molecules were used in our model as molecular markers of vesicular secretion in indirect quantification of vesicle fractions, following the methodology for indirect vesicle quantification recently described by our group (Oliveira et al., 2010). This analysis (Figure 6) revealed that the *grasp* mutant showed a regular pattern of vesicular extracellular release, in comparison to WT cells. GXM concentration was

reduced in vesicle fractions from the mutant, suggesting that GRASP is required for packaging GXM into secretory vesicles.

Although secretion of capsular components is required for capsule assembly, it was recently demonstrated that capsule enlargement also requires polysaccharide molecules with higher effective diameters (Frases et al., 2009). We then analyzed this parameter in GXM fractions from WT and mutant cells (Figure 7). Profiles of diameter distribution of GXM isolated from culture supernatants of WT and complemented cells were very similar. Values of diameter for polysaccharide fractions obtained from cultures of the grasp mutant, however, were below the detection limit under the conditions used in this study. Since interaction with divalent cations is known to promote GXM aggregation and to increase the dimensions of polysaccharide molecules (Nimrichter et al., 2007; Frases et al., 2008) we incubated the extracellular GXM samples with 1 mM $CaCl_2$ for 1 h to favor detection of smaller molecules. Under these conditions, extracellular GXM fractions from mutant cells lacking GRASP expression reached dimensions that were within the limit of detection in our analyses. These fractions, however, were still smaller in diameter than samples produced by WT and reconstituted cells. This observation suggests that the *grasp* mutant is in fact less effective in secreting larger GXM molecules.

Discussion

Fungal cells use a number of unconventional secretion mechanisms to release proteins to the extracellular space (Albuquerque et al., 2008; Rodrigues et al., 2008a; Panepinto et al., 2009; Duran et al., 2010; Manjithaya et al., 2010). In addition to the well characterized unconventional secretory pathways, it has been proposed that fungi exploit a vesicular pathway for the trans-cell wall passage of molecules to the extracellular milieu (Rodrigues et al., 2007; Nosanchuk et al., 2008; Rodrigues et al., 2008a; Rodrigues et al., 2008b; Oliveira et al., 2009; Casadevall et al., 2009). Genes and proteins involved in such mechanisms, however, are only partially known. Polysaccharides, lipids and pigments can also be extracellularly secreted by fungi (Rodrigues et al., 2007; Eisenman et al., 2009), but the pathways required for these processes remain largely unknown. GXM biosynthesis in *C. neoformans*, in fact, illustrates this scenario. Many of the enzymes required for synthesis of this polysaccharide have been described (reviewed in (Doering, 2009)), but several aspects related to its cellular distribution and traffic remain unknown. Within the cell, GXM is associated to the membranes of still unknown organelles (Oliveira et al., 2009). Outside the cell, GXM is found either in its free form, as a soluble polysaccharide (reviewed in (Zaragoza et al., 2009)), or associated to exosome-like structures (Rodrigues et al., 2007; Rodrigues et al., 2008a). Defects in GXM production and capsule assembly led to avirulent phenotypes in *C. neoformans* (reviewed in (Zaragoza et al., 2009)). Since GXM production and extracellular release are crucial for cryptococcal pathogenesis, this is clearly an area of active study.

A few examples of cryptococcal genes implicated in GXM traffic and secretion are available in the literature, including *CAP59, SAV1* and *SEC6*. *CAP59* gene encodes a 458-amino-acid protein of unknown function that shows homology to *CMT1*, whose product shows alpha-1,3-mannosyltransferase activity (Chang et al., 1995; Sommer et al., 2003). Deletion of *CAP59* resulted in acapsular cells with increased diameters (Garcia-Rivera et al., 2004), which was attributed to intracellular polysaccharide accumulation. In fact, a missense mutation of *CAP59* partially hampered the trafficking of GXM, but not proteins (Garcia-Rivera et al., 2004). The existence of cellular pathways required for GXM export was further confirmed in studies focused on the role of post-Golgi secretion events in *C. neoformans*. The products of *SAV1* and *SEC6*, which correspond, respectively, to a putative vesicle-associated Rab GTPase and to a member of the post-Golgi exocytic complex, were demonstrated to be involved in vesicle-mediate export of GXM to the surface of *C. neoformans* (Yoneda and Doering, 2006; Panepinto et al., 2009). GXM is then released to the extracellular space in vesicles that traverse the cell wall (Rodrigues et al., 2007). Therefore, extracellular GXM release is linked to elements of the conventional, post-Golgi secretory pathway (Hu et al., 2007; Panepinto et al., 2009) and to exosome-like structures (Rodrigues et al., 2007), but the involvement of other cellular pathways in the traffic of the polysaccharide are largely unknown.

In *S. cerevisiae* and *P. pastoris*, GRASP is involved in unconventional secretory mechanisms that require autophagy genes, early endosomal compartments, and MVBs (Duran et al., 2010; Manjithaya et al., 2010). The

possibility raised by many authors that GXM secretion could involve such organelles (Takeo et al., 1973a, b; Yoneda and Doering, 2006; Nosanchuk et al., 2008; Oliveira et al., 2009; Rodrigues et al., 2008a; Rodrigues et al., 2008b; Casadevall et al., 2009) combined to the emerging roles of GRASP in unconventional secretory pathways (Kinseth et al., 2007; Levi and Glick, 2007; Schotman et al., 2008; Nickel and Rabouille, 2009; Cabral et al., 2010; Duran et al., 2010), we asked whether this protein would regulate GXM secretion and virulence in *C. neoformans*.

In comparison to WT and reconstituted cells, the *grasp* mutant of *C. neoformans* was less efficient in killing lethally infected mice. The explanation for this finding is probably related to its reduced ability to secrete GXM and to form regular capsules. Since the polysaccharide is believed to cause many deleterious effects to the host (reviewed in (Zaragoza et al., 2009)), a reduction in the extracellular concentration of GXM would favor the host defense and infection control. Remarkably, GXM obtained from the *grasp* mutant showed altered physical chemical properties (reduced dimensions), although it was regularly recognized by an antibody raised to the polysaccharide. The fact that the *grasp* mutant produced smaller GXM fibers is consistent with the observation of a reduced capsule. Capsule enlargement was demonstrated to be linked to the availability of polysaccharide molecules of increased dimensions (Frases et al., 2009).

Based on studies involving monoclonal antibodies, it is generally accepted that first steps of GXM synthesis occur intracellularly (Feldmesser et al., 2001; Garcia-Rivera et al., 2004; Yoneda and Doering, 2006; Oliveira et al.,

2009). However, it is not known whether polysaccharide molecules distributed to intracellular organelles show the same structural features observed in extracellular fractions. For instance, antibody-binding GXM small precursors could be synthesized in intracellular compartments to be then transferred to the extracellular space, where polymerization would occur by aggregative mechanisms (Nimrichter et al., 2007; Frases et al., 2009). In this case, the reduced secretion of GXM by the *grasp* mutant would be simply related to problems with cellular traffic, as described for other systems (Kinseth et al., 2007; Duran et al., 2010; Manjithaya et al., 2010). GXM polymerization would be favored in conditions where the polysaccharide is more abundant (Nimrichter et al., 2007), and this might explain why the effective diameter of GXM fibers is reduced in supernatants of the *grasp* mutant. Alternatively, if GXM polymerization and fiber enlargement is an intracellular event, the reduced concentration of GXM in extracellular vesicle fractions could indicate a role for GRASP in packaging larger polysaccharide molecules into secretory vesicles. The methods that are currently available for the analysis of intracellular GXM still do not discriminate larger fibers from the smaller ones, so this question still remains unanswered.

GRASP is required for unconventional secretion in *D. discoideum*, *S. cerevisiae*, *P. pastoris* and *D. melanogaster* (Kinseth et al., 2007; Duran et al., 2010; Manjithaya et al., 2010). Although it is well accepted that GRASP is a Golgi-associated protein, it has also been proposed that the protein could mediate vesicle fusion events at the plasma membrane (Nickel and Rabouille, 2009). This proposal is in agreement with the fact that GRASP is localized at

the plasma membrane during epithelial cell remodeling in *D. melanogaster* (Schotman et al., 2008). Remarkably, *D. discoideum* cells lacking GRASP show defects in the stage of final fusion of vesicles required for unconventional secretion with the plasma membrane (Cabral et al., 2010). In contrast to what have been demonstrated for a *S. cerevisiae grasp* mutant (Oliveira et al., 2010), *C. neoformans* cells lacking this protein showed normal extracellular release of vesicles. This observation could suggest that, in this organism, GRASP is required for GXM loading into secretory vesicles rather than the release of these structures to the extracellular space, although we still do not have any evidence that GXM and GRASP interact. Alternatively, the role played by GRASP in polysaccharide traffic could be related to its presence in Golgi cisternae, which also requires experimental confirmation.

Protein secretion has been extensively explored in bacterial and eukaryotic cells. Although polysaccharide secretion in fungi was described many decades ago, the mechanisms that regulate are largely unexplored. To our knowledge, this study is the first to demonstrate a role for GRASP in polysaccharide secretion, as well as in microbial virulence. The observation that GRASP regulates a process required for the pathogenesis of *C. neoformans* adds a new function to the list of the important roles played by this protein in the biology of eukaryotic organisms.

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Figures and legends

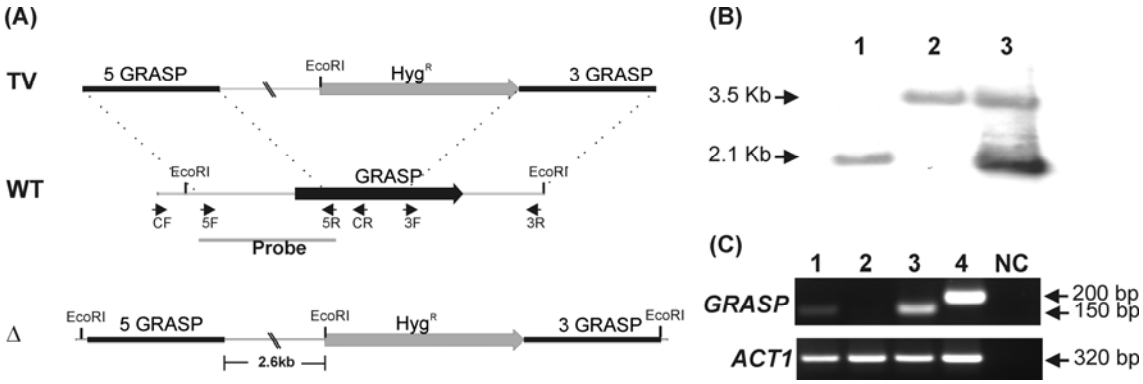

Figure 1. Deletion and complementation of the *C. neoformans GRASP*. A. Scheme for the construction of the *grasp* knockout strain. *GRASP* gene was replaced with the hygromicin resistant marker (Hyg^R) cassette (gray box). GRASP 5' and 3' flanks (5 GRASP and 3 GRASP, respectively) were fused with Hyg^R cassette by Delsgate methodology (Garcia-Pedrajas et al., 2008). The resulting targeting vector (TV) was used for *C. neoformans* transformation. The wild type locus of GRASP (WT) and the position of primers utilized for GRASP gene disruption are also indicated. The black bar scale corresponds to 500 base pairs (bp). The cleavage sites of EcoRI restriction enzime are indicated in the deletion scheme. B. Southern blot analysis. Genomic DNA (10µg) from WT (lane 1), *grasp* mutant (lane 2) and *grasp::GRASP* reconstituted (lane 3) strains were digested with EcoRI restriction enzyme. The 5' gene flank was used as probe in Southern hybridization. Left numbers (in base pairs) indicate the hybridization signal sizes based upon the position of molecular size marker. **C**. Semi-quantitative RT-PCR using cDNA from WT (lane 1), *grasp* (lane 2) and *grasp::GRASP* (lane 3) strains as template. Right numbers (in bp) indicate the length of the transcript amplification for *GRASP* (upper panel) and *ACT1* (lower

panel) genes. Lane 4: positive control using genomic DNA as template. NC: negative control of the PCR.

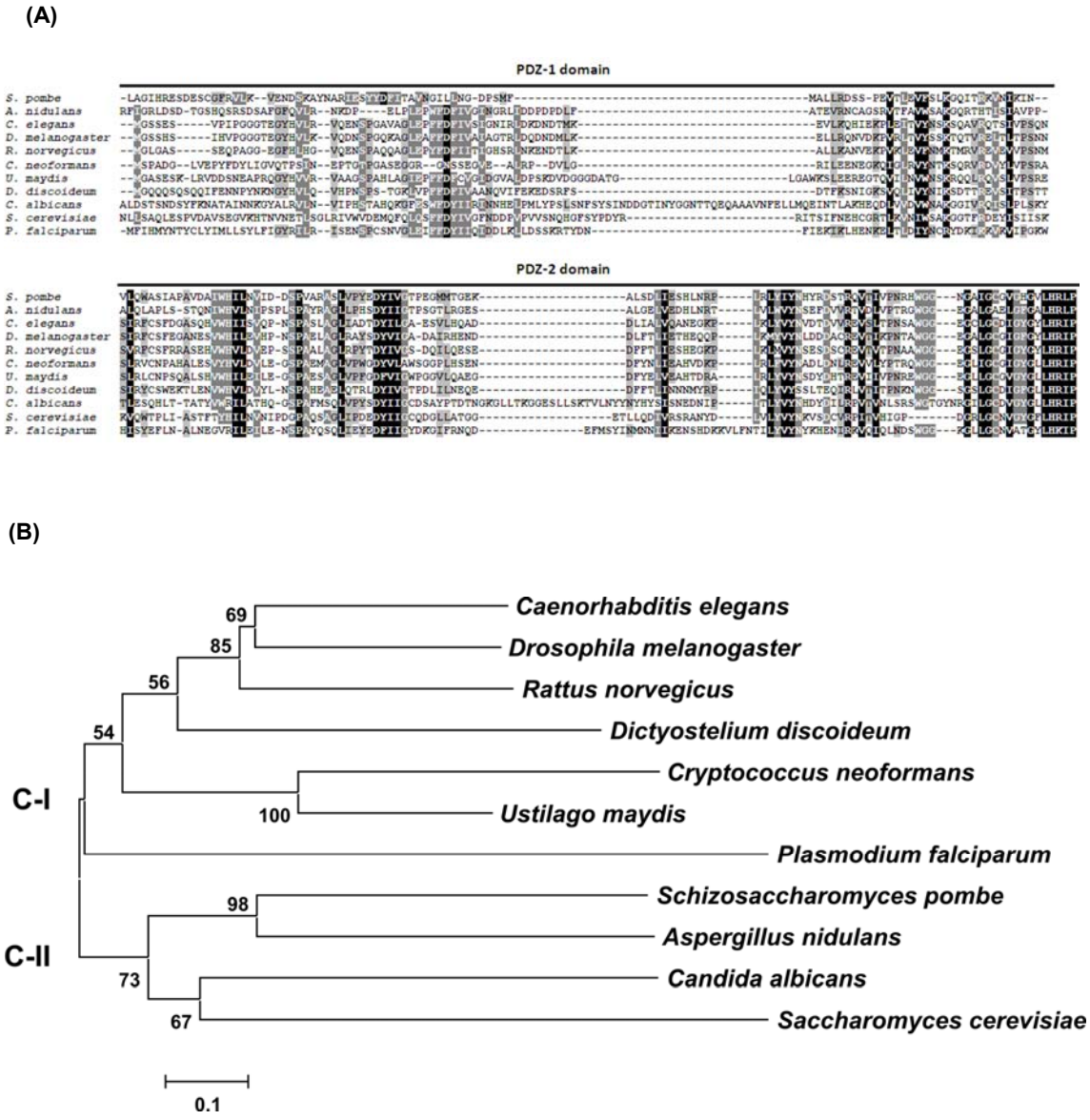

Figure 2. *In silico* analysis of the *C. neoformans* GRASP ortholog. A. Alignment of the GRASP PDZ domains (PDZ-1 and PDZ-2) from *R. norvegicus* (Genbank accession number AAB81355.2), *C. elegans* (Genbank accession number NP_501354), *D. melanogaster* (Genbank accession number AAF49092), *S. cerevisiae* (Genbank accession number NP_010805), *D. discoideum* (Genbank accession number EAL60823), *S. pombe* (Genbank accession number NP_593015.1), *P. falciparum* (Genbank accession number AAN35366), *A. nidulans* (Broad Institute accession number ANID_11248), *U.*

maydis (Broad Institute accession number UM01076), *C. albicans* (Broad Institute accession number CAWG_01021) and *C. neoformans* (Broad Institute accession number CNAG_03291) using ClustalX2. B. Phylogenetic analysis applying the Neighbor-Joining method including GRASP sequences from distinct eukaryotic organisms listed above. The phylogeny tree splits into two major clades. C-I and C-II represents clades I and II, respectively. The bar marker indicates the genetic distance, which is proportional to the number of amino acid substitutions.

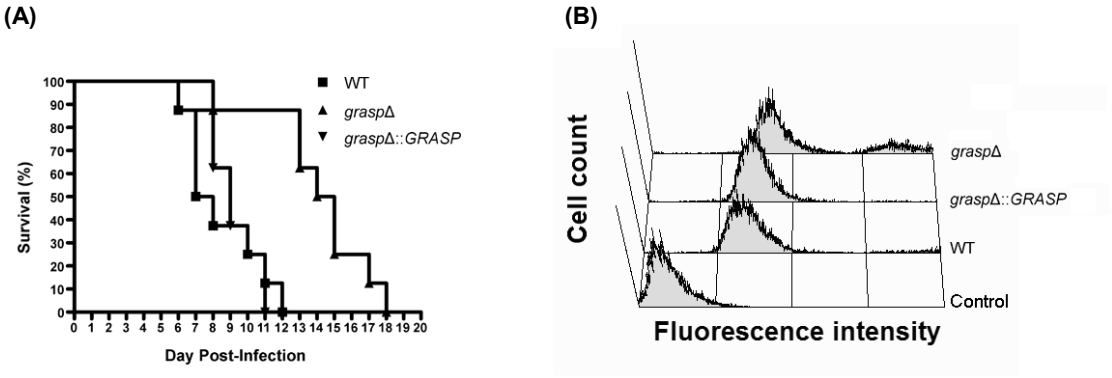

Figure 3. A. The *grasp* mutant show attenuated virulence in an animal model of cryptococcosis. Mice were lethally infected with *C. neoformans* for daily monitoring of survival. Animals infected with the *grasp* mutant strain survived significantly longer (P<0.01). B. Association of FITC-labeled *C. neoformans* cells with murine phagocytes. The similarity in the fluorescence levels of macrophages after infection with WT, *grasp* and reconstituted cells is indicative of similar indices of association between fungal and host cells.

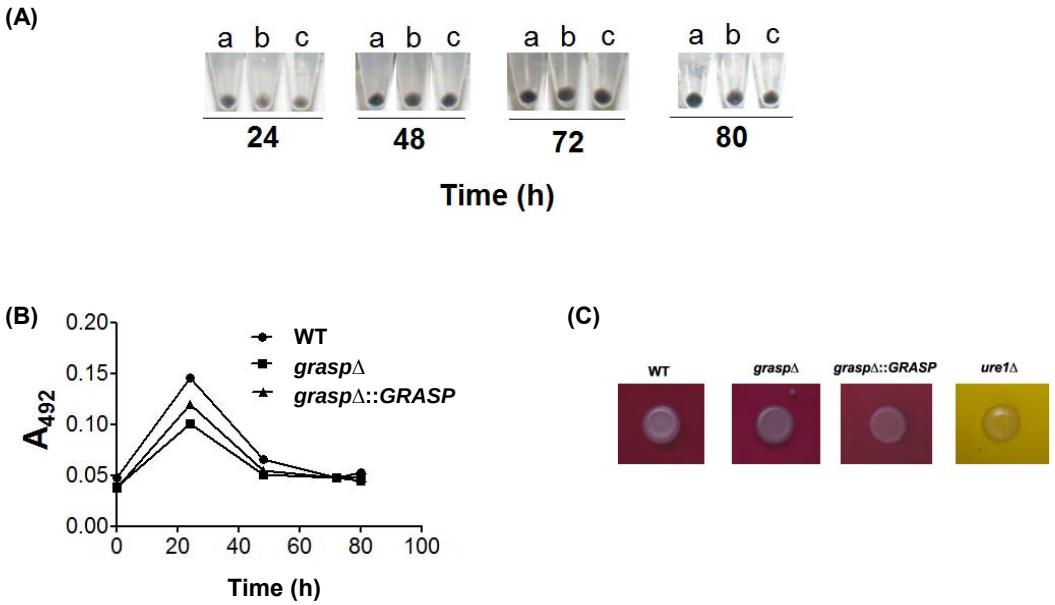

Figure 4. Lack of GRASP expression does not affect pigmentation (A-B) or urease activity (C) in cryptococcal cultures. A. Pigmentation of *C. neoformans* cells after growth in the presence of L-DOPA. Pigmented pellets of WT (a), *grasp* (b) and reconstituted cells (c) are shown. B. Release of pigment-related molecules into *C. neoformans* cultures. C. Urease activity was clearly detected (pink color) in cultures of WT, *grasp* and reconstituted cells, but not in cultures of a urease deletion mutant (*ure1*Δ, yellow).

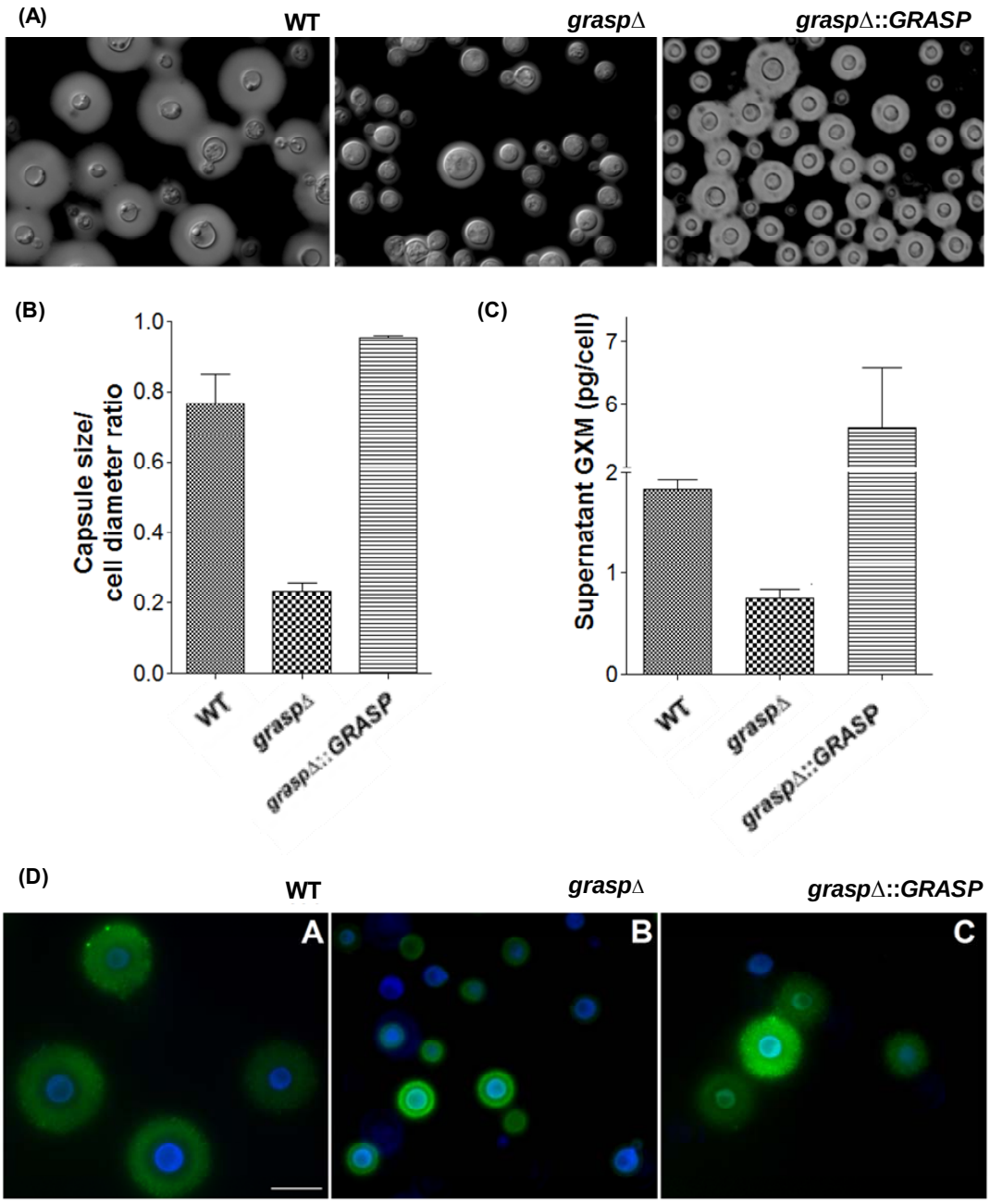

Figure 5. GRASP is required for normal GXM secretion and capsule assembly. A. India ink counterstaining of *C. neoformans* cells. Yeast strains are indicated on the top of each panel. B. Determination of capsule size of the *C. neoformans* cells illustrated in A. C. GXM determination in culture supernatants, showing that *C. neoformans grasp* mutant show a reduced content of extracellular GXM. D. Reactivity of *C. neoformans* cells with calcofluor white (blue fluorescence)

and a monoclonal antibody raised to GXM (green fluorescence). A, WT strain; B, *grasp* mutant strain; C, complemented strain. Scale bars in (A) and (D) represent 20 and 10 μm, respectively.

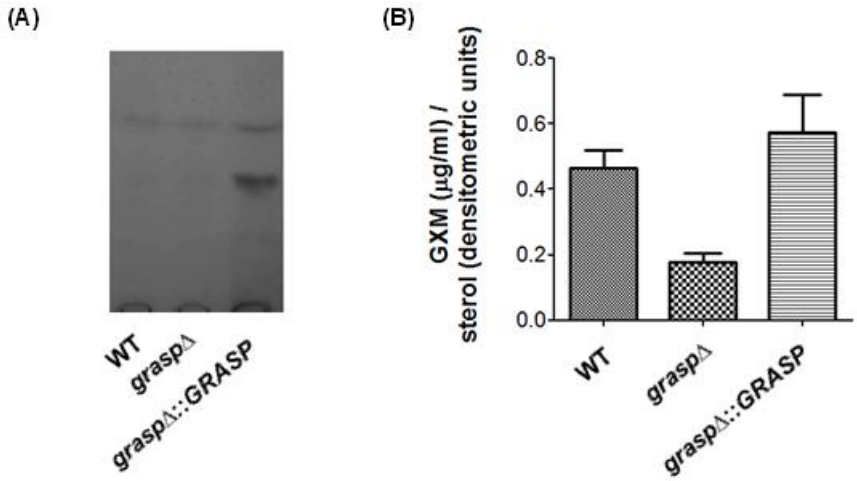

Figure 6. Sterol and GXM determination in extracellular vesicle fractions from WT, *grasp* and reconstituted *C. neoformans* cells. The similarity in sterol contents, determined by TLC (A), suggests equivalent vesicle secretion in all strains. The content of GXM in fractions from the *grasp* mutant (B) was significantly reduced in comparison with both WT and reconstituted strains (P<0.001 in both cases).

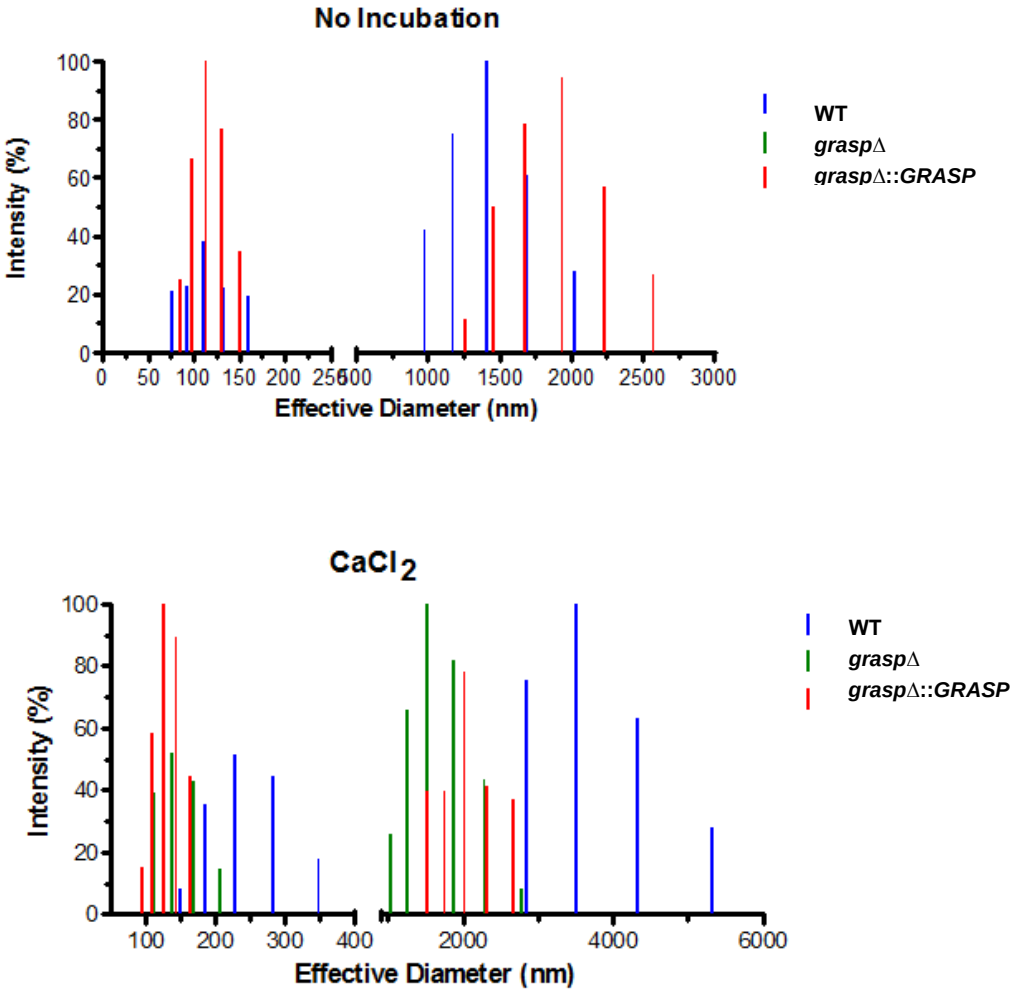

Figure 7. Effective diameter determination of GXM fibers obtained from cultures of WT, *grasp*, and complemented cells. The upper panel shows diameter determination under regular conditions of GXM analysis. The lower panel illustrates diameter determination after incubation of polisaccharide fractions with 1 mM $CaCl_2$.

Table 1. **List of primers used in this study.**

Primer name	Sequence (5'-3')	Purpose
CnGRASPF	AAAATAGGGATAACAGGGTAATGAGATACCAGATGGACTGAA	Disruption construct for *GRASP1*, 5' flank
CnGRASP5R	GGGGACAAGTTTGTACAAAAAAGCAGGCTATATATTCTGCCCAGCACATCT	Disruption construct for *GRASP1*, 5' flank
CnGRASP3F	GGGGACCACTTTGTACAAGAAAGCTGGGTAATGCTAATGTGAAACGCAAT	Disruption construct for *GRASP1*, 3' flank
CnGRASP3R	AAAAATTACCCTGTTATCCCTAGTAACGAGAAGTGCTGTCTC	Disruption construct for *GRASP1*, 3' flank
GRASPcompF	ATAATGCAATGCGATGGCTGC	Amplification of *GRASP* for complementation
GRASPcompR	AATCCCCTCAAGAGCTCACGG	Amplification of *GRASP* for complementation
RTGRASPF	AGTTCTTTACCCTACTAGACAG	Amplification of *GRASP* for RT-PCR
RTGRASPR	TCTCCTCACATTGTCAGATTC	Amplification of *GRASP* for RT-PCR
RTACTF	CCTTCTACGTCTCTATCCAG	Amplification of *ACT1* for RT-PCR
RTACTR	TTTCAAGCTGAGAAGACTGG	Amplification of *ACT1* for RT-PCR

6. Discussão

A pandemia da AIDS contribuiu significativamente para o aumento de pesquisas científicas relacionadas à biologia do patógeno oportunista *C. neoformans*. A disponibilidade de inúmeras ferramentas para estudos de função gênica, aliada as recentes publicações de sequências completas dos genomas de distintas linhagens deste microrganismo têm permitido a identificação de genes relevantes para a virulência durante a interação de *C. neoformans* com o hospedeiro humano (WARNOCK, 2006; MA & MAY, 2009).

Distintos aspectos da biologia de *C. neoformans* foram abordados na presente Tese. Os resultados obtidos neste trabalho contribuíram para a identificação de um novo componente da via de sinalização controlada por calcineurina, para a caracterização de um fator de transcrição que regula o metabolismo de nitrogênio, e para a descrição de uma proteína que está envolvida na secreção de GXM e montagem da cápsula polissacarídica em *C. neoformans*.

A via de sinalização regulada por calcineurina é fundamental para a adaptação de *C. neoformans* ao hospedeiro durante o processo de infecção. Um dos principais fatores de virulência deste fungo, a habilidade de desenvolvimento a 37°C, é diretamente controlada por esta via de sinalização, sendo a disponibilidade de cálcio indispensável para a ativação das proteínas calmodulina e calcineurina (KOZUBOWSKI *et al.*, 2009). Além disto, processos como *mating*, frutificação monocariótica e integridade da parede celular são igualmente regulados por esta via, enfatizando a sua relevância em importantes e distintos aspectos da biologia de *C. neoformans* (CRUZ *et al.,*

2001; FOX *et al.,* 2001; KRAUS *et al*., 2003). Em *S. cerevisiae*, a via de sinalização controlada por Ca^{2+}/calcineurina regula o ciclo celular e as respostas à limitação de glicose e ao estresse salino. Nos fungos filamentosos *N. crassa* e *M. grisea*, esta via está envolvida na regulação de ciclo celular, na germinação de esporos e no desenvolvimento de hifas (ZELTER *et al.*, 2004).

Os transportadores de cálcio presentes em vacúolos de células fúngicas são responsáveis pela regulação dos níveis de cálcio citosólico e pelo transporte do referido metal para esta organela de armazenamento (ZELTER *et al.*, 2004). O transportador Vcx1 de *C. neoformans*, assim como o seu ortólogo em *S. cerevisiae* (PITTMAN *et al.*, 2004), são regulados negativamente por calcineurina. Em ensaios de interação com macrófagos alveolares foi constatado que Vcx1 é importante para a sobrevivência de *C. neoformans* durante o processo de fagocitose. Este fato pode estar associado à diminuição da secreção de GXM extracelular pelo mutante de *C. neoformans vcx1Δ*, uma vez que há inúmeras evidências que apontam que o polissacarídeo GXM altera a função dos macrófagos em múltiplos níveis (ZARAGOZA *et al.*, 2009). Ensaios de infecção em modelo experimental comprovaram que o transportador de cálcio vacuolar Vcx1 atua no processo de virulência de *C. neoformans.* O mesmo foi observado para os transportadores de cálcio de membrana e de retículo endoplasmático de *C. neoformans*, Cch1 e Eca1, respectivamente (LIU *et al.*, 2006; FAN *et al.*, 2007). Estes resultados ressaltam a importância da sinalização mediada por cálcio durante a interação com o hospedeiro.

O transporte e metabolismo de fontes de nitrogênio são essenciais para o desenvolvimento de fungos patogênicos. Durante a infecção, distintos nichos do hospedeiro com disponibilidade variável destas fontes são colonizados pelo patógeno, o que justifica a presença de vias de regulação especializadas para controlar o metabolismo de nitrogênio nestes microrganismos (MARZLUF, 1997). O fator de transcrição Gat1 em *C. neoformans* é responsável pela ativação de genes sensíveis ao processo de repressão catabólica por nitrogênio. A virulência de *C. neoformans* não é influenciada pela ação de Gat1, divergindo do que está descrito para os ortólogos em *C. albicans* e *A. fumigatus (*HENSEL *et al.,* 1998*;* LIMJINDAPORN *et al.,* 2003*).*

A expressão de genes relacionados à biossíntese do polissacarídeo GXM, o principal componente da cápsula em *C. neoformans,* é controlada por Gat1. Este fato pode justificar os níveis reduzidos de GXM secretados no sobrenadante de cultivos do mutante *gat1 in vitro*. Além disto, genes envolvidos na síntese de ergosterol, metabolismo de ferro e integridade da parede celular são regulados por Gat1 em *C. neoformans*. Há evidências de que a expressão de Gat1 é controlada pelo fator de transcrição Sre1 de *C. neoformans* em condições de hipóxia (LEE *et al.*, 2007). Sre1 atua também na regulação de genes envolvidos na biossíntese de ergosterol, no metabolismo de ferro e nitrogênio, e na integridade da parede celular, o que indica que os fatores de transcrição Sre1 e Gat1, de alguma forma, estão relacionados e atuam em paralelo na regulação de importantes aspectos da biologia de *C. neoformans*.

A secreção de fatores de virulência é um mecanismo de patogenicidade utilizado por *C. neoformans* para o estabelecimento da infecção. Este

microrganismo utiliza estratégias de secreção não convencionais para a liberação de vesículas contendo polissacarídeos e proteínas no meio extracelular (RODRIGUES *et al.*, 2008). Sabe-se que as funções do complexo de Golgi estão relacionadas à síntese e à secreção de GXM em *C. neoformans*, baseado em análises funcionais dos genes *SAV1* e *SEC6*, ambos envolvidos em vias de secreção de vesículas mediadas por tal organela (YONEDA & DOERING, 2006; PANEPINTO *et al.*, 2009). Há evidências de que a proteína GRASP está envolvida em mecanismos de secreção não convencionais em distintos organismos eucarióticos (KINSETH *et al.*, 2007; SCHOTMAN *et al.*, 2008; NICKEL & RABOUILLE, 2009; CABRAL *et al.,* 2010; DURAN *et al.,* 2010; MANJITHAYA *et al.,* 2010). Em *C. neoformans*, esta proteína participa nos processos de síntese da cápsula polissacarídica, de secreção de GXM e de virulência. Divergindo do que ocorre em *S. cerevisiae* (DURAN *et al.,* 2010), a produção de vesículas não é alterada em mutantes *grasp* de *C. neoformans*, entretanto os níveis de GXM presentes nestas estruturas secretadas são significativamente menores.

Considerando fatores de *C. neoformans* no processo de interação patógeno-hospedeiro, a cápsula polissacarídica desponta como o principal fator de virulência envolvido na patogênese deste fungo. A síntese da cápsula em *C. neoformans* é regulada pela ação de pelo menos 23 genes, os quais estão envolvidos em distintos processos biológicos, representando assim a sua complexidade. Os polissacarídeos capsulares podem estar ancorados na parede celular, formando uma barreira física, ou podem ser secretados para o meio extracelular. Uma série de estudos indica que ambas as formas conferem

efeitos benéficos para *C. neoformans* durante a interação com o hospedeiro. O principal polissacarídeo que compõe a cápsula, GXM, induz sérios efeitos deletérios no sistema imune do hospedeiro, o que contribui significativamente para o estabelecimento da infecção (ZARAGOZA *et al.*, 2009). Neste contexto, é importante ressaltar a relevância da caracterização funcional de Vcx1, Gat1, e GRASP em *C. neoformans*, os quais participam do processo de secreção de GXM para o meio extracelular. Além disto, o transportador de cálcio Vcx1 e a proteína GRASP também estão envolvidos no processo de patogenicidade em *C. neoformans*.

Estudos de genômica funcional contribuem para o melhor entendimento das interações de *C. neoformans* com o hospedeiro, e buscam auxiliar no alcance do principal desafio para a comunidade científica da área: a determinação de novos alvos para o desenvolvimento de vacinas e antifúngicos eficientes para o tratamento da criptococose.

7. Conclusões

- O transportador de cálcio vacuolar Vcx1 de *C. neoformans* é regulado pela proteína calcineurina e atua na sobrevivência de *C. neoformans* durante a fagocitose por macrófagos alveolares.

- O transportador de cálcio Vcx1 participa do processo de virulência em condições de interação de *C. neoformans* com o hospedeiro.

- O fator de transcrição Gat1 regula a expressão de genes sensíveis à repressão catabólica por nitrogênio e de genes envolvidos na síntese de ergosterol, metabolismo de ferro, integridade da parede celular e síntese da cápsula polissacarídica em *C. neoformans*.

- O fator de transcrição Gat1 de *C. neoformans* não é requerido para os processos de virulência ou sobrevivência durante a interação com macrófagos alveolares.

- O transportador de cálcio Vcx1 e o fator de transcrição Gat1 de *C. neoformans* participam do processo de secreção de GXM para o meio extracelular.

- A proteína GRASP está envolvida na secreção do polissacarídeo capsular GXM e na montagem da cápsula em *C. neoformans*.

- A proteína GRASP influencia o diâmetro das fibras do polissacarídeo capsular GXM em *C. neoformans*.

- A proteína GRASP atua na virulência de *C. neoformans* em condições de infecção.

8. Perspectivas

Definir um papel para GRASP de *C. neoformans* na secreção do polissacarídeo GXM *in vivo*.

Correlacionar a função de GRASP no processo de secreção não convencional com os níveis extracelulares do polissacarídeo GXM em *C. neoformans*.

Confirmar a localização vacuolar de Vcx1 em *C. neoformans*.

Avaliar a participação de Vcx1 de *C. neoformans* no transporte e estocagem de cálcio nos vacúolos.

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