UNIVERSIDADE DE BRASÍLIA
FACULDADE DE MEDICINA
PROGRAMA DE PÓS-GRADUAÇÃO EM PATOLOGIA MOLECULAR

Fontes preferenciais e mecanismos moleculares envolvidos na homeostase de ferro em *Paracoccidioides* spp.

Tese de Doutorado

Candidata: Elisa Flávia Luiz Cardoso Bailão
Orientadora: Dra. Célia Maria de Almeida Soares

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Tese apresentada ao Programa de Pós Graduação em Patologia Molecular, da Faculdade de Medicina, da Universidade de Brasília, como requisito parcial para obtenção do título de Doutor em Patologia Molecular

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O saber a gente aprende com os mestres e os livros. A sabedoria se aprende é com a vida e com os humildes.

Cora Coralina

O início da sabedoria é a admissão da própria ignorância. Todo o meu saber consiste em saber que nada sei.

Sócrates

Aos meus grandes amores e incentivadores...

À Deus, o único conhecedor de todas as minhas angústias, medos e anseios. Nos momentos mais difíceis, o Senhor me carregou nos braços... Obrigada por ser meu companheiro diário...

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SUMÁRIO

LISTA DE ABREVIATURAS

aRNA – ácido ribonucleico (RNA) antisentido;

ATMT – transformação mediada por *Agrobacterium tumefaciens*;

BHI – infuso de cérebro e coração;

BPS – ácido dissulfônico da batofenantrolina;

EBP – proteína de ligação a estrogênio;

EDTA – ácido etilenodiamino tetra-acético;

EROs – espécies reativas de oxigênio;

GPI – glicosilfosfatidilinositol;

MCF – família de carreadores mitocondriais;

MMcM – meio McVeigh-Morton modificado;

mRNA – ácido ribonucleico (RNA) mensageiro;

nanoUPLC-MS^E – cromatografia líquida de alta performance em nano-escala acoplada à espectrometria de massas com aquisições de dados independentes;

*Pb*01 – linhagem 01 de *Paracoccidioides* spp.;

*Pb*18 – linhagem 18 de *Paracoccidioides* spp.;

*Pb*339 – linhagem 339 de *Paracoccidioides* spp.;

PCM – paracoccidioidomicose;

PS2 – espécie filogenética 2;

PS3 – espécie filogenética 3;

PS4 – espécie filogenética 4;

PtII – composto contendo Pt^{2+};

qRT-PCR – reação em cadeia da polimerase (PCR) quantitativa (q) utilizando a enzima transcritase reversa (RT);

S1 – espécie 1;

SC – meio sintético completo;

Zn-PPIX – protoporfirina IX ligada a zinco;

RESUMO

Paracoccidioides spp. é um fungo termodimórfico, causador da paracoccidioidomicose (PCM), uma doença endêmica da América Latina. Atualmente o gênero *Paracoccidioides* é considerado um grupo filogenético, contendo duas espécies distintas: *P. brasiliensis* e *P. lutzii*. Porém, todos os isolados identificados até o momento são capazes de causar a PCM. O ferro é um micronutriente essencial para todos os eucariotos, pois participa como cofator de diversas vias metabólicas. Porém, dentro do hospedeiro, a quantidade de ferro disponível para captação pelo patógeno é limitada. As fontes de ferro do hospedeiro preferenciais de *Paracoccidioides* spp. e os mecanismos moleculares utilizados pelo fungo para captar ferro ainda não foram investigados. Neste trabalho, foi constatado um robusto crescimento de *Paracoccidioides* spp. na presença de hemina ou hemoglobina, corroborado pela capacidade do fungo de internalizar uma protoporfirina e de lisar hemácias. Análises transcricionais e proteômicas apontaram para a indução de possíveis receptores de hemoglobina na presença desta fonte de ferro e para a possível internalização de toda a molécula de hemoglobina pelo fungo. Um desses possíveis receptores, Rbt5, foi caracterizado neste trabalho. Rbt5 é uma proteína GPI-ancorada presente na superfície de *Paracoccidioides* sp., capaz de se ligar à hemina, protoporfirina e hemoglobina e apresenta propriedades antigênicas. Uma linhagem silenciada para o gene *rbt5* foi obtida através de RNA antisentido e de transformação mediada por *Agrobacterium tumefaciens*. Esta linhagem apresentou menor sobrevivência dentro de macrófagos e em baço de camundongo, indicando que Rbt5 pode ser importante para o estabelecimento da infecção por *Paracoccidioides* spp. Além disso, a via redutiva de captação de ferro foi investigada neste fungo. Análises *in silico* apontaram que *Paracoccidioides* spp. possa utilizar redutases férricas, ferroxidases e transportadores de ferro e zinco para captar ferro livre ou ligado a certas moléculas, como a transferrina. Os genes codificantes para essas moléculas possivelmente envolvidas com a captação de ferro foram ativados na ausência desse metal, o que aponta para a importância dessa via em condições de privação de ferro. Genes envolvidos com a homeostase intracelular de ferro também foram investigados neste trabalho e apresentaram-se regulados pelo metal. A atividade de redutase férrica foi demonstrada para *Paracoccidioides* sp., porém ensaios de captação com ^{59}Fe apontaram que a via redutiva só é funcional em condições de privação do metal e que as redutases férricas de *Paracoccidioides* spp. não são fortemente afetadas por platina. Moléculas de superfície envolvidas com a captação de ferro pelo fungo podem ser importantes alvos de vacinas e/ou de drogas antifúngicas. Dessa forma, este trabalho pode contribuir para o desenvolvimento de terapias alternativas para o tratamento da PCM.

ABSTRACT

Paracoccidioides spp. is a thermodimorphic fungus, which causes paracoccidioidomycosis (PCM), an endemic disease in Latin America. Currently, the *Paracoccidioides* genus is considered a phylogenetic group containing two distinct species: *P. brasiliensis* and *P. lutzii*. However, all isolates identified to date are able to cause PCM. Iron is an essential micronutrient for all eukaryotes, since it participates as a cofactor in several metabolic pathways. However, the amount of available iron inside the host is limited to the pathogen. The host iron sources that are used by *Paracoccidioides* spp. and the molecular mechanisms used by the fungus to acquire iron has not been investigated. In this study, a robust growth of *Paracoccidioides* spp. in the presence of hemin or hemoglobin was observed. This fact was supported by the ability of the fungus to internalize protoporphyrin rings and to lyse erythrocytes. Transcriptional and proteomic analysis indicated the induction of possible hemoglobin receptors in the presence of hemoglobin and the possibility of internalization of the entire molecule by the fungus. One of these potential receptors, Rbt5, was characterized in this work. Rbt5 is a GPI-anchored protein, present at the *Paracoccidioides* sp. cell surface. The protein is able to bind hemin, hemoglobin and protoporphyrin and presents antigenic properties. A *Paracoccidioides* sp.*rbt5*knockdown strain was obtained in this work using antisense RNA and *Agrobacterium tumefaciens*-mediated transformation. This strain showed lower survival inside macrophages and in mouse spleen, indicating that Rbt5 may be important for the establishment of *Paracoccidioides* spp. infection. Furthermore, the reductive iron uptake pathway was investigated in this fungus. *In silico* analysis pointed that *Paracoccidioides* spp. could use ferric reductases, ferroxidases and zinc and iron transporters to uptake free iron or iron bound to certain molecules, such as transferrin. The genes encoding those molecules possibly involved in iron uptake were activated in the absence of the metal, which points to the importance of this pathway in conditions of iron deprivation. Genes involved in intracellular iron homeostasis were also investigated in this work and presented regulation by the metal. A ferric reductase activity was demonstrated for *Paracoccidioides* sp.; however, ^{59}Fe uptake assays showed that the reductive pathway is functional only in conditions of iron deprivation and that the *Paracoccidioides* spp. ferric reductases are not strongly affected by platinum. Surface molecules involved in iron uptake by the fungus may be important targets for vaccines and/or antifungal drugs. Thus, this work can contribute to the development of alternative therapies for PCM treatment.

Capítulo 1

Aspectos gerais

I. INTRODUÇÃO

I.1. O fungo *Paracoccidioides* spp.

Os fungos dimórficos são caracterizados pela mudança de uma fase filamentosa multicelular para uma unicelular, quando infectam os tecidos do hospedeiro. Essa é uma propriedade genética intrínseca de fungos como *Histoplasma capsulatum*, *Blastomyces dermatitidis*, *Coccidioides immitis*, *Coccidioides posadassi*, *Sporothrix schenckii*, *Penicillium marneffei* e *Paracoccidioides spp.* (RAPPLEYE; GOLDMAN, 2006). A patogenicidade dos mesmos parece estar relacionada ao dimorfismo, pelo fato de linhagens incapazes de transitar morfologicamente não serem virulentas (MARESCA; KOBAYASHI, 2000). Em contrapartida, a transição morfológica não parece prejudicar a perpetuação destes organismos, uma vez que eles podem sobreviver no solo como saprobiotas e crescer como micélio. Em *Paracoccidioides spp.* esse dimorfismo está fortemente associado à mudança de temperatura sofrida pelo fungo ao penetrar o hospedeiro (SAN-BLAS; NINO-VEGA; ITURRIAGA, 2002) e por esse motivo ele é classificado como um fungo termodimórfico. Dessa maneira, as duas fases deste fungo podem ser cultivadas *in vitro* apenas variando-se a temperatura de 36 ^{o}C, quando o fungo tem um aspecto de levedura, para temperaturas inferiores a 28 ^{o}C, quando se tem a fase miceliana (BAGAGLI et al., 2006).

Comparações filogenéticas de dermatófitos e fungos dimórficos baseadas na subunidade ribossomal 28S classificaram *Paracoccidioides spp.* como pertencente ao reino Fungi, filo Ascomycota, classe Plectomyceto, ordem Onygenales, família Onygenaceae, juntamente com *B. dermatitidis* e *H. capsulatum* (LECLERC; PHILIPPE; GUEHO, 1994; SAN-BLAS; NIÑO-VEGA, 2008). *Paracoccidioides brasiliensis* era considerada espécie única de seu gênero até 2006. Com a popularização da sistemática molecular, foi proposta a existência de pelo menos quatro diferentes clados: S1 (espécie 1, com 38 isolados), PS2 (espécie filogenética 2, com 6 isolados), PS3 (espécie filogenética 3, com 21 isolados) (MATUTE et al., 2006) e PS4 (espécie filogenética 4, com 5 isolados identificados até o momento) (BOCCA et al., 2013). S1 é a espécie melhor distribuída geograficamente, ocorrendo no Brasil, Argentina, Paraguai, Uruguai, Peru e Venezuela. PS2 ocorre no Brasil e na Venezuela. PS3 é um grupo geograficamente restrito à Colômbia (MATUTE et al., 2006; THEODORO et al., 2012) **(Figura 1)**. PS4 foi descrita recentemente e contém isolados clínicos provenientes da Venezuela, porém ainda é pouco caracterizada (BOCCA et al., 2013). Análises filogenéticas posteriores revelaram que a linhagem 01 de *Paracoccidioides* spp. (*Pb*01) distancia-se dos quatro clados descritos anteriormente, sugerindo que *Pb*01 seja uma nova espécie

do gênero *Paracoccidioides* (CARRERO et al., 2008). Com o estudo de mais isolados de *P. brasiliensis* foi identificado um grupo com 17 isolados similares genotipicamente, incluindo *Pb*01, que se distanciam do grupo S1/PS2/PS3/PS4 (BOCCA et al., 2013), reforçando que o grupo "semelhante a *Pb*01" refere-se a uma nova espécie. Esta espécie foi denominada *Paracoccidioides lutzii*, em homenagem a Adolfo Lutz, o primeiro a isolar e descrever este fungo em 1908 (TEIXEIRA et al., 2009; TEIXEIRA et al., 2013b). Considera-se que *P. lutzii* seja endêmico da região Centro-Oeste brasileira, mais especificamente dos Estados de Goiás e Mato Grosso (TEIXEIRA et al., 2009). Porém, isolados provenientes de Rondônia foram recentemente classificados como *P. lutzii* baseado em *loci* genealógicos (TEIXEIRA et al., 2013b) **(Figura 1)**.

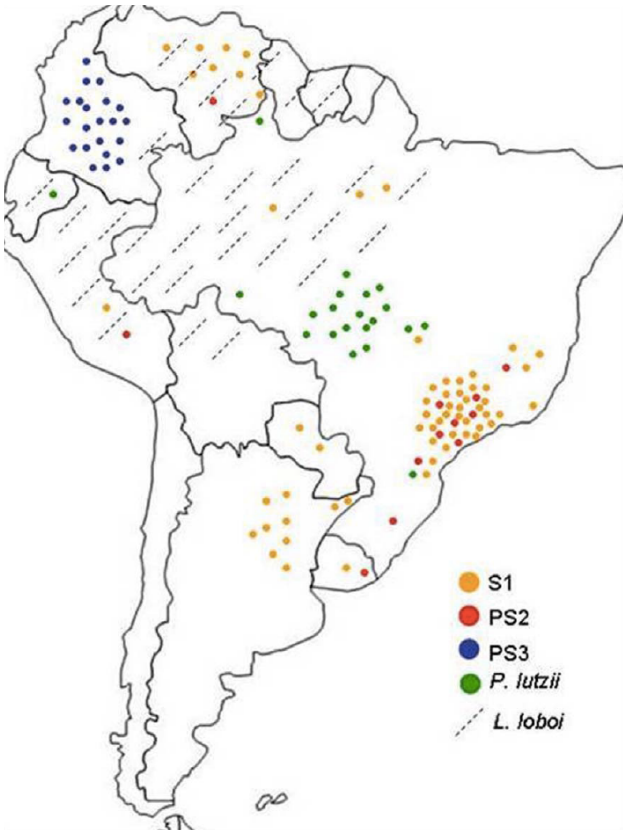

Figura 1. Distribuição geográfica de *Paracoccidioides* spp. S1 ocorre no Brasil, Argentina, Paraguai, Uruguai, Peru e Venezuela. PS2 e *P. lutzii* ocorrem no Brasil principalmente. E PS3 é um grupo geograficamente restrito à Colômbia. *Lacazia loboi* (*L. loboi*) é uma espécie irmã de *Paracoccidioides* spp., fonte de estudo do trabalho de onde a imagem foi extraída (THEODORO et al., 2012).

Ainda não há um consenso se S1, PS2, PS3 são variantes geográficas de uma mesma espécie ou se são espécies distintas (THEODORO et al., 2012). Por meio de análises filogenéticas, sugere-se que S1/PS2/PS3, juntamente com *P. lutzii*, são isolados reprodutivamente na natureza (MATUTE et al., 2006; TEIXEIRA et al., 2009). O isolamento reprodutivo ou genético é o primeiro passo na divergência de espécies fúngicas (TAYLOR et al., 2000). Dessa forma, a busca por diferenças entre as espécies do gênero *Paracoccidioides* é crescente, visto que isso pode influenciar no diagnóstico e no tratamento da paracoccidioidomicose (PCM), micose sistêmica causada por esse gênero.

Morfologicamente, as espécies do gênero *Paracoccidioides* são bastante similares. Macroscopicamente, a fase de levedura de *Paracoccidioides* spp. é caracterizada por colônias rugosas, amareladas e de aspecto cerebriforme **(Figura 2A)**. Microscopicamente, são visualizadas células esféricas ou ovais de 2-30 μm de diâmetro, multinucleadas, apresentando paredes espessas e birrefringentes, aparentemente duplas, e citoplasma contendo vacúolos de lipídeos **(Figura 2B)**. O diagnóstico histológico da PCM baseia-se no achado de estruturas fúngicas com formato de "roda de leme" que refletem células-mães em brotamento multipolar envolvidas por células-filhas periféricas (RESTREPO, 1978). Já a fase miceliana, apresenta-se macroscopicamente como colônias pequenas, irregulares, esbranquiçadas ou acastanhadas **(Figura 2C)**. Microscopicamente, as hifas são finas (1-3 μm), hialinas e septadas, apresentando conídios uninucleados terminais ou intercalares (QUEIROZ-TELLES, 1994) **(Figura 2D)**. A única característica morfológica potencialmente capaz de distinguir *P. brasiliensis* de *P. lutzii* é a forma e o tamanho do conídio. Esta última espécie parece possuir conídios muito longos em comparação com os conídios de *P. brasiliensis* (TEIXEIRA et al., 2009; THEODORO et al., 2012; TEIXEIRA et al., 2013b).

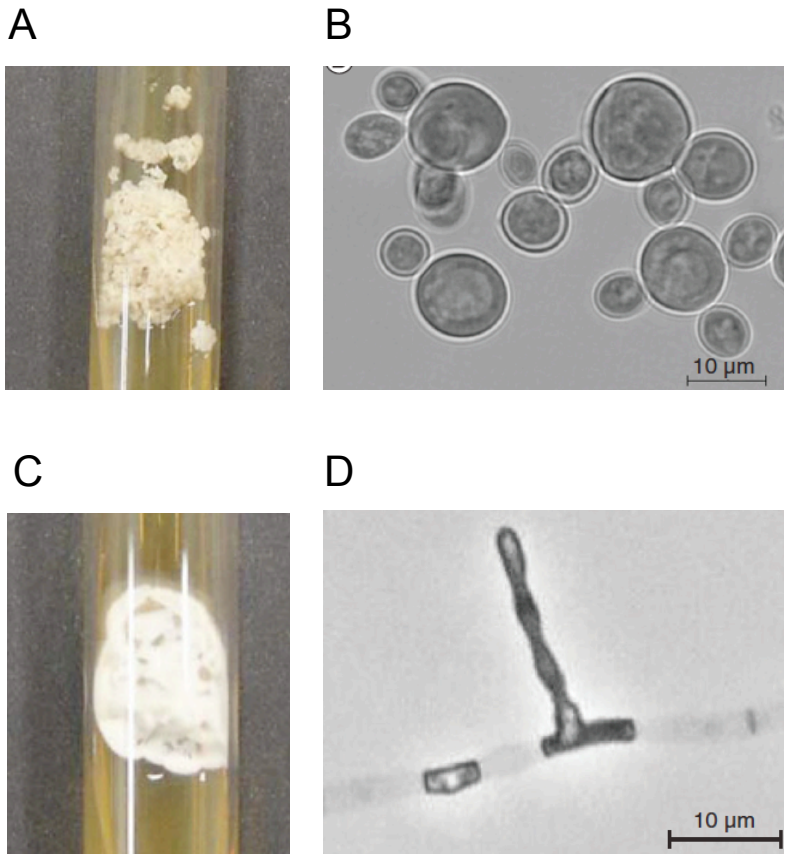

Figura 2. Aspecto morfológico de Paracoccidioides spp. A. Macroscopicamente, a fase de levedura de *Paracoccidioides* spp. é caracterizada por colônias rugosas, amareladas e de aspecto cerebriforme. **B.** Microscopicamente, as células leveduriformes são esféricas ou ovais, multinucleadas, com aspecto de roda de leme. **C.** Macroscopicamente, a fase miceliana apresenta-se como colônias pequenas, irregulares, esbranquiçadas ou acastanhadas. **D.** Microscopicamente, as hifas são finas, hialinas e septadas, apresentando conídios uninucleados terminais ou intercalares. Adaptado de Bocca e colaboradores (2013) e de foto disponível *online* (http://www.parasitologiaclinica.ufsc.br/index.php/info/conteudo/doencas/micoses/pacacoccidioidomicose/).

Genomicamente, diferentes linhagens de *P. brasiliensis* são mais semelhantes entre si do que estas em relação a *P. lutzii*. Os genomas de *Pb*18 (S1) e de *Pb*03 (PS2) possuem cerca de 30 Mb cada um e o genoma de *Pb*01, representando *P. lutzii*, possui cerca de 33 Mb. A similaridade de sequências é maior entre *Pb*18 e *Pb*03 (~96%) do que entre esses e *Pb*01 (~90%). Os transposons representam 8-9% dos genomas de *Pb*18 e *Pb*03 e 16% do genoma de *Pb*01 (DESJARDINS et al., 2011). O número total de genes, por sua vez, não é capaz de diferenciar entre as duas espécies, variando de 7.610 para *Pb*03 a 8.130 para *Pb*01 (DESJARDINS et al., 2011).

Por outro lado, um estudo proteômico comparativo apontou que não há diferenças bem marcadas entre as duas espécies do Gênero *Paracoccidioides*, mas sim entre diferentes isolados dos quatro grupos filogenéticos: *P. brasiliensis*S1, PS2, PS3 e *P. lutzii* (PIGOSSO et al., 2013). A β-oxidação demonstrou ser bastante induzida em *Pb*339, representante do grupo S1, indicando que a oxidação de ácidos graxos seja importante para fornecer acetil-CoA para o metabolismo central de carbono deste grupo filogenético. As estratégias metabólicas mais induzidas em *Pb*2, representante de PS2, são a via das pentoses-fosfato e a degradação de aminoácidos, que também podem alimentar o metabolismo central de carbono do fungo. *Pb*Epm83, representante de PS3, parece utilizar preferencialmente carboidratos para as rotas aeróbicas de produção de energia. *Pb*01, representando *P. lutzii*, parece utilizar um metabolismo mais anaeróbico para a produção de energia a partir de carboidratos. Isso foi proposto a partir da observação da alta produção de enzimas envolvidas com a glicólise e a fermentação alcoólica e o alto nível de etanol nesta linhagem (PIGOSSO et al., 2013). Mais isolados do Gênero *Paracoccidioides* precisam ser investigados para o desenvolvimento de marcadores bioquímicos capazes de distinguir entre os quatro grupos do complexo *Paracoccidioides*. Dessa maneira, a análise de polimorfismos de nucleotídeo único (SNPs) é a forma mais utilizada ainda para distinguir entre os diferentes grupos do complexo *Paracoccidioides* (THEODORO et al., 2012).

Os nichos ecológicos de *Paracoccidioides* spp. ainda não estão completamente esclarecidos, porém sugere-se que o fungo tenha pelo menos dois. O primeiro corresponde ao seu estado filamentoso, em que o fungo vive saprobioticamente na natureza, já tendo sido isolado de solo, de água e de plantas (RESTREPO; MCEWEN; CASTANEDA, 2001). O segundo refere-se à sua associação com animais homeotérmicos, inclusive o homem, em sua fase de levedura, podendo levar à doença. Devido ao pequeno número de isolamentos de *Paracoccidioides* spp. do solo, acredita-se que este ambiente não seja o hábitat permanente do patógeno, vivendo ele tempo variável neste local. Presume-se que o fungo *Paracoccidioides* spp. ocorra normalmente em ambientes úmidos, próximos a rios, onde possa ser protegido por representantes de espécies aquáticas como moluscos, anfíbios, peixes e artrópodes. Estes reservatórios aquáticos estariam fornecendo ao parasito nutrientes, umidade, competição biológica limitada e temperatura apropriada para a sobrevivência do fungo no meio ambiente. A infecção natural do fungo em alguns animais silvestres, como tatus (*Dasypus novemcinctus*, *Dasypus septemcinctus* e *Cabassous centralis*), e animais domésticos, como cachorros (*Canis familiares*), já foi reportada. Entretanto, estes

organismos são considerados hospedeiros acidentais e não reservas naturais do fungo, pois os mesmos podem ser acometidos pela doença (CONTI-DIAZ, 2007).

Estudos de genômica funcional em *Paracoccidioides* spp. são relativamente recentes porque pouco se sabe sobre sistemas de recombinação homóloga no fungo e um estágio sexual de *Paracoccidioides* spp. não é bem caracterizado. O primeiro estudo que sugeriu a existência de um ciclo sexual em *Paracoccidioides* spp. foi publicado em 2005, quando se identificou possíveis proteínas envolvidas com o tipo de acasalamento (Mat-1 e Mat-2) em análises transcritômicas (FERNANDES et al., 2005). Posteriormente, foram identificados no genoma de *Paracoccidioides* spp. vários genes possivelmente envolvidos com desenvolvimento sexual, meiose e esporulação, altamente conservados em relação a outros fungos dimórficos (DESJARDINS et al., 2011). Recentemente, a primeira evidência da formação inicial de ascocarpos, estruturas derivadas da reprodução sexuada, de *Paracoccidioides* spp.*in vitro* foi publicada. Porém não foi observada a produção de cleistotécio ou ascos mesmo após um ano de co-cultivo de isolados de tipos reprodutivos opostos (TEIXEIRA et al., 2013a).Caso o ciclo sexual de *Paracoccidioides* spp. exista naturalmente, sugere-se que ele ocorra durante a fase saprofítica, miceliana, do fungo (TEIXEIRA et al., 2013a).

Dessa forma, essa carência de dados compromete o desenvolvimento de técnicas eficientes de genética clássica para o trabalho com *Paracoccidioides* spp. Então, as estratégias mais utilizadas para modular a expressão de genes alvo em *Paracoccidioides* spp. são a tecnologia do RNA antisentido (aRNA) e a transformação mediada por *Agrobacterium tumefaciens* (ATMT) (ALMEIDA et al., 2009; RUIZ et al., 2011; TORRES et al., 2013). O princípio básico da tecnologia do aRNA envolve a degradação de um RNA mensageiro (mRNA) específico pela formação de um RNA dupla fita (RAMACHANDRAN; IGNACIMUTHU, 2013). Dessa forma, é inserido no genoma do fungo um oligonucleotídeo capaz de gerar um RNA complementar ao RNA de estudo para silenciar a expressão do gene de interesse. A forma mais eficiente de fazer a inserção desse oligonucleotídeo, já demonstrada para *Paracoccidioides* spp., é a ATMT. A ATMT é uma técnica de mutagênese insercional baseada nas propriedades naturais de infecção da bactéria fitopatogênica *A. tumefaciens*. Essa bactéria é capaz de transferir randomicamente seu DNA para o genoma do hospedeiro (WOOD et al., 2001).

I.2. A paracoccidioidomicose

Paracoccidioides spp. alcança o hospedeiro usualmente através da via respiratória, por inalação de propágulos de micélio, como conídios. Nos pulmões, com o aumento da temperatura para em torno de 36 ^{o}C, esses propágulos convertem-se para a fase de levedura, de onde podem se disseminar para diferentes órgãos e tecidos, pelas vias hematogênica e/ou linfática (FRANCO, 1987). Essa mudança brusca no momento da infecção requer um ajuste rápido e coordenado por parte do fungo para manter a sobrevivência dele e para capacitá-lo a invadir o hospedeiro. As mudanças morfogenéticas em fungos dimórficos são reversíveis, sendo consideradas uma forma de adaptação oportunista às condições do hospedeiro. Além disso, a conversão para a forma de levedura é um requisito para a progressão da infecção (SAN-BLAS; NINO-VEGA; ITURRIAGA, 2002; SAN-BLAS; NIÑO-VEGA, 2008).

A susceptibilidade do hospedeiro a *Paracoccidioides* spp. está relacionada tanto a fatores do fungo, principalmente à virulência do isolado e à quantidade de inóculo, quanto do próprio hospedeiro (FRANCO, 1987). Já foi demonstrado que o hormônio sexual feminino 17β-estradiol inibe seletivamente a transição de micélio para levedura em *Paracoccidioides* spp. (RESTREPO et al., 1984). Foi observada também a participação deste hormônio na resistência de fêmeas de rato ao desenvolvimento inicial da PCM (ARISTIZABAL et al., 2002). Uma proteína de ligação a estrogênio (EBP) de massa molecular de 60 kDa foi identificada no citosol tanto das células leveduriformes quanto da fase miceliana do fungo, sugerindo que ao entrar em contato com o hormônio, forma-se um complexo proteína-ligante, que inibe a conversão morfológica e, portanto, o estabelecimento e a disseminação da doença (STOVER et al., 1986; CLEMONS; FELDMAN; STEVENS, 1989). As análises do perfil transcricional de *Paracoccidioides* spp. detectaram uma expressão preferencial do gene que codifica para a EBP na fase patogênica (FELIPE et al., 2005). Essas evidências permitem afirmar uma resistência inata de fêmeas à PCM, demonstrada pela taxa de 10 a 15 homens para 1 mulher que são acometidos pela PCM. Esta prevalência não é observado na infância, onde a infecção e a doença distribuem-se uniformemente entre ambos os sexos (SHIKANAI-YASUDA et al., 2006).

A PCM é uma micose sistêmica, endêmica da América Latina, distribuída do México à Argentina (RESTREPO; MCEWEN; CASTANEDA, 2001). Dentro da região endêmica, estima-se

que 10% da população esteja infectada com o fungo, porém a maioria dela não apresenta a forma ativa da doença (BRUMMER; CASTANEDA; RESTREPO, 1993). Se esta estimativa estiver correta, a prevalência dessa infecção fúngica é comparável a da doença de Chagas na América Latina (MARTINEZ, 2010). Em um estudo de detecção do antígeno paracoccidioidina por reação intradérmica, revelou-se que a PCM infecção é mais comumente observada em trabalhadores rurais e fazendeiros entre 30 e 40 anos de idade (RESTREPO-MORENO, 1994). Acredita-se que o trabalho com solo e plantações em área rural é fator ocupacional predisponente para a aquisição da PCM (FRANCO, 1987). A PCM é raramente observada em crianças e adultos jovens (3 e 10% dos casos relatados, respectivamente) e é mais frequentemente diagnosticada em homens adultos entre 30 e 60 anos de idade (BRUMMER; CASTANEDA; RESTREPO, 1993).

Por não ser uma doença de notificação compulsória, o impacto da PCM nas áreas endêmicas é de difícil quantificação e, talvez por isso, possua baixa visibilidade como um problema de saúde pública, o que enquadra a PCM na lista de doenças negligenciadas (MARTINEZ, 2010). Porém, apenas o Hospital Universitário da Faculdade de Medicina de Ribeirão Preto recebe, aproximadamente, 40 novos casos da doença por ano (MARTINEZ, 2010). Estima-se que sejam 3.360 novos casos por ano em todo o Brasil (MARTINEZ, 2010)e que a taxa de mortalidade seja de 5 a 27% dentro da população infectada (BROWN et al., 2012). O Brasil é o país com maior número de casos da doença (80%), seguido pela Venezuela e pela Colômbia (BRUMMER; CASTANEDA; RESTREPO, 1993). No Brasil, a PCM representa 51,2% das causas de morte entre as micoses sistêmicas, seguida por criptococose, candidose e histoplasmose. Com relação à idade e sexo, 53,4% dos pacientes portadores da PCM possuem 30-59 anos e 87,9% são do sexo masculino. Os Estados brasileiros mais atingidos são São Paulo, Minas Gerais, Rio de Janeiro, Paraná e Rio Grande do Sul (PRADO et al., 2009).

Alguns casos autóctones já foram diagnosticados na Europa, nos Estados Unidos da América e na Ásia (JOSEPH; MARE; IRVING, 1966; CHIKAMORI et al., 1984; AJELLO; POLONELLI, 1985). Estes relatos possibilitaram a determinação de um período assintomático da PCM (PCM infecção), que é em média 15,3 anos, que ocorre após o contato do paciente com a área endêmica até a manifestação da doença (BRUMMER; CASTANEDA; RESTREPO, 1993). O longo período de latência, característico da PCM, dificulta o processo de determinação precisa do sítio onde a infecção ocorreu (BORELLI, 1972). O controle da infecção depende da resposta imune celular do tipo Th1, caracterizado pela síntese de citocinas que ativam macrófagos e linfócitos T

$CD4^+$ e $CD8^+$, resultando na formação de granulomas compactos. Estas estruturas tentam controlar a replicação e a disseminação do fungo, porém células leveduriformes quiescentes, porém viáveis, podem persistir no interior do granuloma (SHIKANAI-YASUDA et al., 2006). A reativação endógena pode ocorrer dependendo de fatores genéticos e hormonais do próprio hospedeiro, como debilidade por outras patologias, alcoolismo crônico, má nutrição, fumo, imunossupressão e presença/ausência de estrogênio (RESTREPO-MORENO, 2003). Se a infecção não for controlada, a PCM doença se manifesta no paciente.

As formas clínicas da PCM são classificadas em: aguda ou subaguda (juvenil) e crônica ou adulta (FRANCO, 1987). A forma aguda da micose corresponde a 5% dos casos (BOCCA et al., 2013), afeta principalmente crianças e jovens e é a forma mais grave e com pior prognóstico. Seu quadro clínico caracteriza-se por um desenvolvimento rápido e por marcante envolvimento de órgãos como fígado, baço, gânglios linfáticos e medula óssea. Nesta forma, a PCM conduz à perda gradual de resposta imune celular e induz à produção de altos títulos de anticorpos específicos (BRUMMER; CASTANEDA; RESTREPO, 1993). A forma crônica ou adulta representa mais de 90% dos casos, sendo a maioria dos pacientes constituída por homens adultos entre 30 e 60 anos de idade, que trabalharam em áreas de agricultura (BOCCA et al., 2013). Ao contrário da forma aguda, o quadro clínico apresenta um desenvolvimento lento com comprometimento pulmonar evidente (BRUMMER; CASTANEDA; RESTREPO, 1993). Em aproximadamente 25% dos casos, o pulmão é o único órgão afetado - forma unifocal. Muitas vezes, com o desenvolvimento silencioso da doença, o paciente busca auxílio médico somente quando apresenta sintomas de comprometimento extrapulmonar. Nestes casos se constatam o envolvimento de outros órgãos, como pele, mucosas das vias aéreas superiores, trato digestivo e linfonodos - forma multifocal (LONDERO; RAMOS; MATTE, 1986; BRUMMER; CASTANEDA; RESTREPO, 1993). A forma crônica apresenta notável tendência à disseminação, sendo pouco frequente os quadros onde há somente comprometimento pulmonar (RESTREPO; CANO; TABARES, 1983). O comprometimento do sistema nervoso central é descrito em 6-25% dos pacientes, afetando o córtex, o cerebelo, o cérebro ou todas as regiões (BOCCA et al., 2013). A PCM requer tratamento prolongado, comumente por mais de um ano, com derivados de sulfonas, anfotericina B, azóis ou terbinafina (MARTINEZ, 2010; BOCCA et al., 2013). Recidivas, complicações e sequelas podem acontecer em até 20% dos pacientes. O comprometimento pulmonar pode levar à fibrose e, consequentemente, à insuficiência respiratória (MARTINEZ, 2010).

Todas as espécies do gênero *Paracoccidioides* identificadas até hoje podem causar PCM. Porém várias peculiaridades regionais, ainda não explicadas, já foram relatadas para as manifestações clínicas, o diagnóstico, e o tratamento da doença (TEIXEIRA et al., 2013b). Já foi reportado que no Estado de Goiás existe a presença frequente de uma forma linfática-abdominal da doença, não usual em outras áreas endêmicas (BARBOSA; DAHER; DE OLIVEIRA, 1968). Isso pode ocorrer pela maior frequência de *P. lutzii* em Goiás (TEIXEIRA et al., 2013b). Essa hipótese pode ser corroborada por outro estudo que demonstrou que isolados de *P. lutzii*, diferentemente de isolados de *P. brasiliensis*, causaram peritonite progressiva intensa, seguida de rápida disseminação para baço, fígado, gânglios linfáticos e mesentério intestinal em camundongos (MOLINARI-MADLUM; FELIPE; SOARES, 1999). Além disso, para o diagnóstico da PCM, é comum utilizar testes sorológicos com o antígeno padrão Gp43, purificado da linhagem *Pb*339 do grupo filogenético S1 de *P. brasiliensis*. Porém, esses testes apresentam uma alta taxa de falso negativo quando são utilizados soros de pacientes da região Centro-Norte do Brasil (BATISTA et al., 2010). A utilização de Gp43 de um isolado do grupo filogenético PS3 (Epm83) apresentou resultados semelhantes com soros de pacientes da região Centro-Oeste do Brasil (MACHADO et al., 2013). Com relação ao tratamento da PCM, já foi observado que pacientes infectados com isolados de *P. lutzii* respondem melhor à terapia com a droga trimetoprim-sulfametoxazol do que pacientes infectados com isolados de *P. brasiliensis* (HAHN et al., 2003). Esses dados reforçam a importância do estudo de diferentes isolados de *Paracoccidioides* spp. para aperfeiçoar o diagnóstico e o tratamento da PCM, favorecendo uma melhoria da qualidade de vida do paciente.

I.3. O micronutriente ferro

O ferro, o quarto elemento mais abundante da crosta terrestre, é um íon metálico que se apresenta sob dois estados de valência: Fe^{3+} (estado férrico) ou Fe^{2+} (estado ferroso) (MIETHKE, 2013). Na presença de oxigênio molecular, o ferro é estável como Fe^{3+} e, assim, Fe^{2+} auto-oxida espontaneamente. Dessa forma, o estado férrico é a forma predominante do ferro no ambiente. Porém, Fe^{3+} liga-se tão avidamente aos seus ligantes que torna esses complexos inertes quimicamente (KOSMAN, 2003). Por isso, Fe^{2+} é a forma mais utilizada como parte de grupos prostéticos, como ferro mono e dinuclear, grupo Fe-S e heme (KOSMAN, 2013). Esses grupos prostéticos têm alto potencial óxido-redutor e fazem parte de enzimas envolvidas em uma série de processos biológicos **(Figura 3)**, como biossíntese de DNA e de vitaminas, biossíntese e

degradação de aminoácidos, assimilação de nitrogênio, respiração celular, detoxificação de radicais livres, dentre outros (CANESSA; LARRONDO, 2013; KOSMAN, 2013). Como muito desses processos estão envolvidos com o metabolismo de energia, o ferro é essencial para quase todos os organismos descritos. Porém eles enfrentam dificuldades para captar este micronutriente pela insolubilidade do Fe^{3+} em água em pH neutro e pela competição com os organismos que ocupam o mesmo nicho (KOSMAN, 2013). A maioria dos micro-organismos vivem em condições em que a concentração de ferro solúvel é menor que 10^{-9} M, que é o caso de ambientes aeróbicos, não ácidos ou no interior do hospedeiro eucarioto, no caso de micro-organismos patogênicos (MIETHKE, 2013).

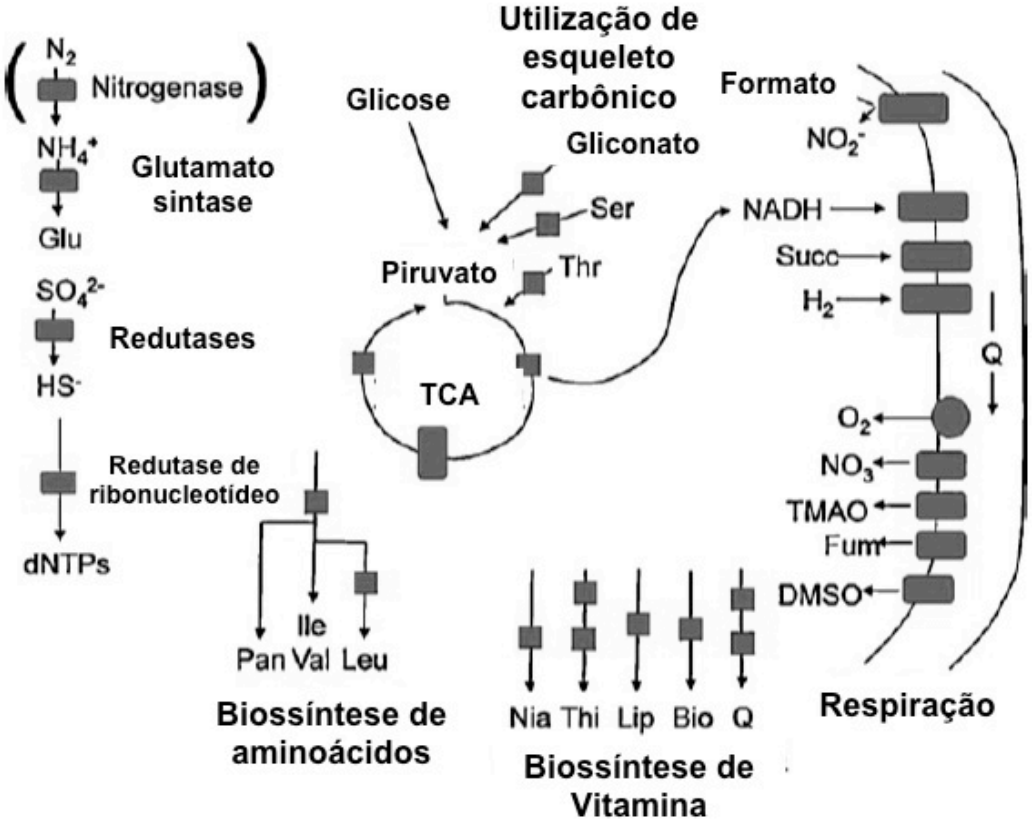

Figura 3. Processos biológicos que contam com a participação do ferro. Várias enzimas, representadas pelas formas geométricas, utilizam ferro como cofator e participam de diversas vias metabólicas. Adaptado de Kosman (2013).

Os fungos utilizam principalmente duas vias para captar o micronutriente ferro. Uma delas envolve a captação de sideróforos ou grupo heme mediada por receptores (KOSMAN, 2013). Sideróforos são pequenas moléculas que atuam como quelantes de Fe^{3+} de alta afinidade (HAAS;

EISENDLE; TURGEON, 2008). E o grupo heme é um anel orgânico heterocíclico, chamado de protoporfirina IX, covalentemente ligado a um átomo de Fe^{2+} (HAMEL et al., 2009). Quando o Fe^{2+} do grupo heme auto-oxida, o complexo porfirina-Fe^{3+} passa a ser chamado de hemina, com concomitante produção de ânions superóxido **(Figura 4)** (PARROW; FLEMING; MINNICK, 2013). A outra estratégia usada por fungos é chamada de via redutiva de assimilação de ferro por envolver a redução do Fe^{3+} para aumentar sua solubilidade. Este passo é seguido pela oxidação e internalização simultâneas do íon (KOSMAN, 2013).

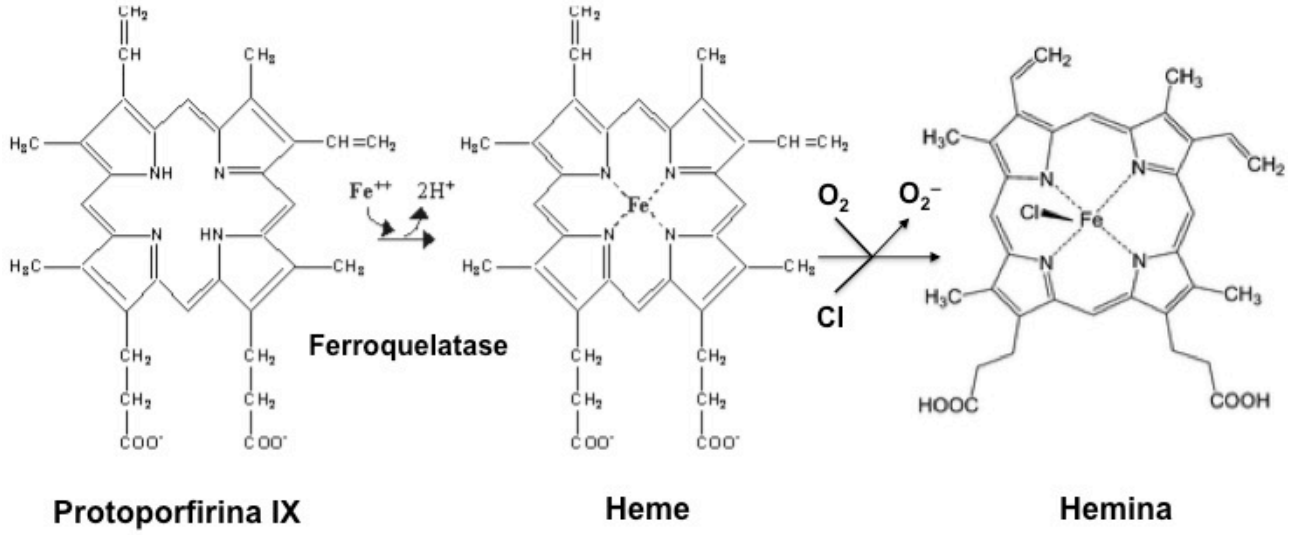

Figura 4. Síntese do grupo heme e da hemina a partir da protoporfirina IX. A adição de Fe^{2+} ao anel de protoporfirina IX, pela enzima ferroquelatase, forma o grupo heme. Quando esta molécula sofre auto-oxidação, ânion superóxido é liberado e a hemina é formada. Adaptado de imagens disponíveis *online* (http://www.rpi.edu/dept/bcbp/molbiochem/MBWeb/mb2/part1/heme.htm e http://www.rsc.org/ej/PP/2013/c2pp25247c/c2pp25247c-f1.gif).

A aquisição de ferro pelos fungos deve ser estritamente regulada porque ainda não foi descrito um mecanismo de excreção desse micronutriente nesses micro-organismos (KAPLAN; KAPLAN, 2009) e o ferro em excesso é tóxico, já que participa das reações de Fenton, gerando espécies reativas de oxigênio (EROs), como radicais hidroxil (PROUSEK, 2007). Esses radicais podem degradar polissacarídeos, causar rupturas nas fitas de DNA, inativar enzimas e iniciar a peroxidação de lipídeos (MCCORD, 1996). Quando o ferro está em excesso dentro da célula fúngica, genes codificantes para proteínas envolvidas com mecanismos de captação de ferro têm sua expressão reprimida; enquanto que genes que participam da estocagem intracelular de ferro têm

sua expressão aumentada. Contrariamente, quando o ferro está limitado, a expressão de genes que participam da captação de ferro e da mobilização de ferro dos estoques está ativada; enquanto que a expressão de genes codificantes para várias enzimas não essenciais que usam ferro como cofator, como por exemplo componentes da via de biossíntese de grupo heme, é reprimida (JUNG; KRONSTAD, 2008; VERGARA; THIELE, 2008; HAAS, 2012).

A competição por ferro entre o patógeno e o hospedeiro é um aspecto fundamental da virulência de fungos (JUNG et al., 2008). Para reduzir a quantidade acessível aos micro-organismos, o ferro do hospedeiro não está disponível no sangue, em fluidos extracelulares ou no interior da célula, estando predominantemente ligado a proteínas ligantes de ferro de alta afinidade, como a transferrina (soro), a hemoglobina (sangue), a lactoferrina (muco) e a ferritina (meio intracelular) (BULLEN et al., 2005; KORNITZER, 2009). Esse mecanismo de sequestro de micronutrientes, descrito como "imunidade nutricional", é visto como uma resistência natural a infecções (WEINBERG, 1975). Dessa forma, micro-organismos patogênicos têm desenvolvido mecanismos para acessar o ferro ligado a proteínas do hospedeiro. Porém pouco se sabe sobre as preferências de fungos patogênicos por fontes de ferro do hospedeiro e sobre as estratégias utilizadas pelos fungos para obter esse ferro e, dessa forma, estabelecer a infecção.

II. JUSTIFICATIVA

As infecções fúngicas contribuem significativamente para a mortalidade e a morbidade humanas. Estima-se que um milhão e meio de pessoas morram a cada ano vítimas de infeções fúngicas. Apesar disso, o investimento em pesquisa e em tratamento de doenças fúngicas é pequeno quando comparado com o investimento em outras doenças infecciosas (BROWN et al., 2012). A situação piora quando se trata de micoses endêmicas de países em desenvolvimento. A paracoccidioidomicose (PCM), por exemplo, é uma micose sistêmica, endêmica da América Latina, enquadrada na lista de doenças negligenciadas por possuir baixa visibilidade como um problema de saúde pública (MARTINEZ, 2010). Por não ser uma doença de notificação compulsória, o impacto da PCM para a saúde pública é de difícil quantificação (MARTINEZ, 2010), mas estima-se que sejam 4.000 infecções por ano no Brasil e a taxa de mortalidade seja de 5 a 27% dentro da população infectada (BROWN et al., 2012).

Paracoccidioides spp., causador da PCM, é um fungo termodimórfico que pode infectar o hospedeiro através de propágulos aéreos. Após a transição de micélio para levedura nos pulmões do hospedeiro, o fungo pode se disseminar para diversos órgãos e tecidos através das vias hematogênica e linfática (FRANCO, 1987; BORGES-WALMSLEY et al., 2002). A incubação de *Paracoccidioides* sp. com sangue humano não leva a um aumento na expressão de genes envolvidos com a aquisição de ferro pelo fungo, sugerindo que essa condição não é ferro limitante para o micro-organismo (BAILÃO et al., 2006). Nos tecidos do hospedeiro, como nos pulmões, o fungo pode ser internalizado pelos macrófagos (BRUMMER et al., 1989; VOLTAN et al., 2013). Já foi demonstrado que macrófagos não ativados são permissivos para multiplicação intracelular de *Paracoccidioides* sp. (BRUMMER et al., 1989; MOSCARDI-BACCHI; BRUMMER; STEVENS, 1994) e que a disponibilidade de ferro dentro de monócitos é requerida para a sobrevivência desse fungo (DIAS-MELICIO et al., 2006). Porém, as fontes de ferro do hospedeiro preferenciais de *Paracoccidioides* spp. e os mecanismos moleculares utilizados pelo fungo para captar ferro ainda não foram investigados.

O tratamento disponível para a PCM é longo e pode incluir diversos efeitos colaterais e resistência do patógeno à medicação utilizada. Por isso, deve ser considerado o risco de abandono do tratamento pelo paciente, seguido de recaídas, sequelas e fibrose tecidual, o que pode comprometer a qualidade de vida do indivíduo (BOCCA et al., 2013). Dessa forma, existe uma grande demanda para o desenvolvimento de terapias alternativas menos longas, que sobreponham à

resistência do fungo e que sejam mais seguras para o paciente (BOCCA et al., 2013). Drogas ou vacinas podem ser desenvolvidas contra proteínas fúngicas de superfície envolvidas com a captação de ferro dentro do hospedeiro na tentativa de bloquear a captação desse micronutriente e, então, a proliferação do fungo. Além disso, essas proteínas podem servir de rotas para a introdução de agentes antifúngicos nas células fúngicas (KRONSTAD; CADIEUX; JUNG, 2013). Dessa forma, as proteínas de superfície envolvidas com a ligação ao grupo heme ou com a captação de ferro pela via redutiva podem servir como importantes alvos para o desenvolvimento de vacinas, para o bloqueio da proliferação de *Paracoccidioides* spp. dentro dos fagócitos, ou para a introdução de agentes antifúngicos, através de uma estratégia do tipo "cavalo de Tróia", nas células fúngicas.

III. OBJETIVOS

III.1. Objetivo geral

Determinar as fontes de ferro do hospedeiro preferenciais de *Paracoccidioides* spp. e propor um mecanismo de captação de ferro pela via redutiva e de homeostase intracelular desse micronutriente neste fungo.

III.2. Objetivos específicos

Para alcançar o objetivo geral do trabalho, alguns objetivos específicos foram delineados:

- Avaliar o crescimento de *Paracoccidioides* spp. na presença de transferrina, hemoglobina, ferritina e lactoferrina;
- Verificar a indução ou a repressão de transcritos evolvidos com a captação de uma das fontes de ferro preferenciais, por meio de RT-PCR quantitativa (qRT-PCR) em tempo real;
- Identificar as proteínas que são induzidas ou reprimidas durante o crescimento do fungo na presença da fonte de ferro do hospedeiro preferencial por cromatografia líquida de alta performance em nano-escala acoplada à espectrometria de massas com aquisições de dados independentes (nanoUPLC-MS^E);
- Propor um modelo para a captação e homeostase de ferro em *Paracoccidioides* spp., baseado em busca de homologia *in silico*;
- Verificar se as células leveduriformes de *Paracoccidioides* spp. produzem redutases férricas funcionais, por ensaio qualitativo em meio sólido;
- Verificar a indução ou a repressão de transcritos envolvidos com a homeostase de ferro em *Paracoccidioides* spp. em diferentes concentrações de ferro e tempos de incubação do fungo;
- Avaliar a taxa de captação de ferro radioativo (^{59}Fe) em *Paracoccidioides* spp.

Capítulo 2

Fontes preferenciais de ferro do hospedeiro

I. INTRODUÇÃO

O organismo de um adulto sadio possui cerca de 40-50 mg/kg de ferro (ALVAREZ; FERNANDEZ-RUIZ; AGUADO, 2013). A absorção deste elemento acontece no duodeno proximal, mediante a redução de Fe^{3+} por redutases férricas presentes na borda apical dos enterócitos. Após a redução, a internalização é feita por um transportador de metal divalente chamado Dmt1 ou Nramp2. Neste momento, o ferro pode ser estocado, usado em processos celulares ou deixar a célula pela ferroportina, um transportador da membrana basolateral (CASSAT; SKAAR, 2013). O ferro exportado do enterócito é liberado no plasma e liga-se à transferrina, facilitando o transporte para as células alvo e impedindo a produção de radicais livres catalisados por ferro. Quando a transferrina atinge sua célula alvo, a internalização ocorre por endocitose mediada por receptor. A acidificação do endossomo facilita a liberação do ferro que é utilizado em diversos processos biológicos, dentre eles a biossíntese de hemoglobina, ou, quando em excesso, é estocado pela ferritina **(Figura 5)** (CASSAT; SKAAR, 2013).

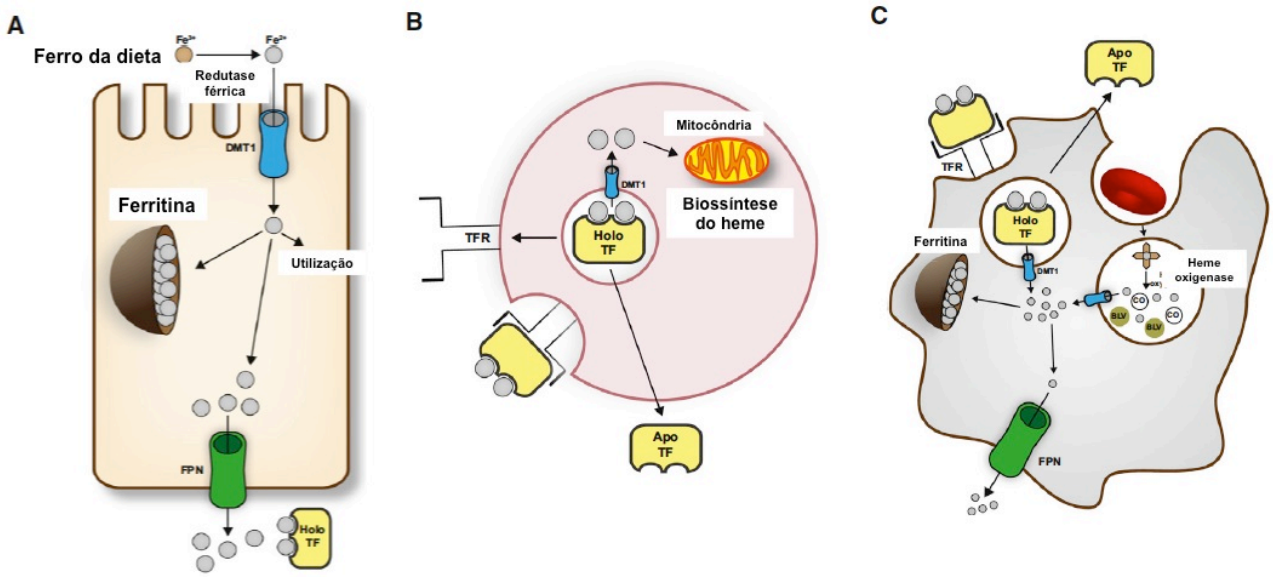

Figura 5. Homeostase de ferro em humanos. A. O ferro da dieta é incialmente reduzido para ser transportado por DMT1 para dentro do enterócito. O Fe^{2+} pode ser utilizado para diversos processos biológicos, ou pode ser estocado pela ferritina, ou ainda deixar a célula pela ferroportina (FPN). O ferro extracelular liga-se à transferrina (Holo TF) para ser transportado a outras células. **B.** Precursores eritróides adquirem ferro da transferrina mediado por um receptor (TFR) via endocitose. O ferro é transportado para o citoplasma pela DMT1 e pode seguir para a mitocôndria, onde será utilizado em reações de biossíntese do grupo heme. **C.** Macrófagos adquirem ferro através do receptor de transferrina (TFR) ou da reciclagem de eritrócitos senescentes. A heme oxigenasse catalisa a degradação do grupo heme a ferro, monóxido de

carbono (CO) e biliverdina (BLV). Posteriormente, o ferro é transportado pela DMT1 para o citoplasma, onde pode ser utilizado para diversos processos celulares, ou estocado na ferritina, ou ainda pode sair da célula pela ferroportina (FPN). Adaptado de Cassat e Skaar (2013).

Menos de 10% da necessidade diária de ferro é obtida pela absorção intestinal. O restante é obtido pela reciclagem do ferro da hemoglobina (HENTZE et al., 2010). Cada eritrócito pode conter 280 milhões de moléculas de hemoglobina, resultando em uma capacidade de ferro de mais de um bilhão de átomos por célula (CASSAT; SKAAR, 2013). A hemoglobina em eritrócitos senescentes é reciclada por macrófagos do sistema reticuloendotelial, principalmente do baço (HENTZE et al., 2010). A enzima heme oxigenase libera ferro e monóxido de carbono do anel de protoporfirina, resultando na produção de biliverdina. O ferro liberado volta para a transferrina no plasma ou é estocado pela ferritina **(Figura 5)** (CASSAT; SKAAR, 2013).

A maior parte do ferro no organismo humano está ligado à hemoglobina (65%) ou à mioglobina (10%) ou faz parte do grupo prostético de diversas enzimas, como catalase, peroxidase ou citocromos (5%). Os 20% restantes do total de ferro do corpo humano encontra-se como reserva intracelular, ligado a proteínas como a ferritina, circula no plasma sanguíneo ligado à transferrina (menos de 1%) ou é excretado em fluidos corporais, como no leite, ligado à lactoferrina. Dessa forma, as concentrações de ferro livre em condições fisiológicas são extremamente reduzidas, na faixa de 10^{-18} M (ALVAREZ; FERNANDEZ-RUIZ; AGUADO, 2013). Para tentar superar essa "imunidade nutricional" imposta pelo hospedeiro (WEINBERG, 1975), muitos micro-organismos desenvolveram mecanismos para utilizar as proteínas ligantes de ferro de alta afinidade (hemoglobina, ferritina, transferrina e lactoferrina) para adquirir esse micronutriente do hospedeiro.

Como explicitado, as fontes de ferro do hospedeiro mais comuns são a hemoglobina e a ferritina. A hemoglobina é uma proteína globular compacta de aproximadamente 64 kDa, composta por quatro subunidades (2 α e 2 β). Cada uma delas possui um grupo prostético heme, protoporfirina IX ligada a um átomo de Fe^{2+}, que se liga reversivelmente ao oxigênio(MARENGO-ROWE, 2006) e, por isso, é utilizada para transportar essa molécula dos pulmões para os tecidos periféricos. Nesses locais, o CO_2, que é abundante, é captado pelas hemácias e metabolizado pela anidrase carbônica a ácido carbônico e H^+. A diminuição do pH favorece a liberação do O_2 do grupo heme. Em contraste, nos pulmões a taxa de CO_2 é mais baixa e o pH é mais alto, favorecendo a ligação do O_2 à hemoglobina (THOM et al., 2013). Já a ferritina é uma proteína formada por 24 subunidades (pesadas, H, e leves, L) e possui cerca de 450 kDa. Essa estrutura proteica possui uma

grande cavidade que comporta até 4500 íons Fe^{3+}(WANG; PANTOPOULOS, 2011). A ferritina tem um papel importante na manutenção da homeostase intracelular de ferro, capturando este metal livre no citoplasma e evitando, assim, as reações de Fenton (TORTI; TORTI, 2002).Apesar de a maioria da ferritina humana ser intracelular, níveis baixos (20-300 μg/l) podem ser encontrados circulando no sangue, mas a função dessa ferritina ainda é desconhecida (HARRISON; ADAMS, 2002).

A transferrina constitui um grupo de glicoproteínas monoméricas de aproximadamente 80 kDa, que se ligam fortemente e reversivelmente a dois íons Fe^{3+} (BOU-ABDALLAH; TERPSTRA, 2012). Um dos membros desse grupo é a própria transferrina humana, que transporta ferro de sítios de absorção para sítios de utilização e estocagem desse micronutriente pelo plasma sanguíneo (FLETCHER; HUEHNS, 1968), porém ela encontra-se apenas 30% saturada com ferro, já que ela pode se ligar a até 40 diferentes íons metálicos (BOU-ABDALLAH; TERPSTRA, 2012). Outro membro desse grupo é a lactoferrina, que está presente em fluidos corporais como leite, saliva e lágrimas, além de ser liberada por neutrófilos durante a degranulação (GONZALEZ-CHAVEZ; AREVALO-GALLEGOS; RASCON-CRUZ, 2009). Apesar de ser uma proteína transportadora de ferro, a lactoferrina é raramente utilizada por micro-organismos como fonte desse micronutriente por possuir atividade antimicrobiana. Essa atividade pode estar associada a dois mecanismos. O primeiro seria a ligação da lactoferrina ao ferro com alta afinidade, o que impediria o micro-organismo de captar o metal ligado à proteína, promovendo o sequestro de ferro em sítios de infecção. O segundo mecanismo seria a interação de aminoácidos carregados positivamente da lactoferrina com moléculas aniônicas da superfície de micro-organismos, o que poderia causar lise celular (GONZALEZ-CHAVEZ; AREVALO-GALLEGOS; RASCON-CRUZ, 2009). A atividade antifúngica de lactoferrina já foi demonstrada para *C. albicans* (KIRKPATRICK et al., 1971; BELLAMY et al., 1993; TAKAKURA et al., 2003) e *Aspergillus fumigatus* (ZAREMBER et al., 2007).

Acredita-se que os fungos possam utilizar três estratégias para captar ferro do hospedeiro. (1) A via redutiva de assimilação de ferro. Esta via conta com redutases férricas de membrana, que estão acopladas a um mecanismo de oxidação-permeação. Esse sistema já foi descrito para *Saccharomyces cerevisiae* (STEARMAN et al., 1996; SINGH et al., 2006), *Candida albicans* (MORRISSEY; WILLIAMS; CASHMORE, 1996; ZIEGLER et al., 2011) e *Cryptococcus neoformans* (JACOBSON; GOODNER; NYHUS, 1998). Em *C. albicans* e em *C. neoformans* essa via tem um importante papel durante a captação de ferro da transferrina (KNIGHT et al., 2005;

JUNG et al., 2008). (2) A captação de biomoléculas utilizando receptores de superfície. Os sideróforos, quelantes de ferro de baixo peso molecular, são reconhecidos por receptores na superfície do fungo e internalizados. Alguns fungos são capazes de produzir, secretar e captar seus próprios sideróforos, como é o caso de *A. fumigatus* (CHARLANG et al., 1981) e *Histoplasma capsulatum* (HOWARD et al., 2000). Outros fungos, porém, são capazes apenas de captar sideróforos produzidos por outros organismos (xenosideróforos), como por exemplo, *S. cerevisiae* (FROISSARD et al., 2007) e *C. neoformans* (JACOBSON; PETRO, 1987). Além disso, sugere-se que alguns fungos consigam internalizar a hemoglobina após a ligação a um receptor de superfície e liberar o ferro intracelularmente, como por exemplo, *C. albicans* (WEISSMAN et al., 2008) e *C. neoformans* (HU et al., 2013). (3) A acidificação do meio circundante. A acidificação torna o ferro mais solúvel e disponível no meio para a captação, como já sugerido para *Neurospora crassa* (HOWARD, 1999). Além disso, alguns fungos podem utilizar a associação dessas estratégias para conseguir captar o ferro do hospedeiro. Um exemplo disso seria a captação de ferritina por *C. albicans*, que utilizaria a adesina e invasina Als3 como receptora desta molécula do hospedeiro. A liberação do ferro da ferritina seria extracelular, promovida pela acidificação do ambiente, e a captação desse micronutriente seria facilitada pela via redutiva (ALMEIDA et al., 2008).

Um dos mecanismos de aquisição de ferro de moléculas do hospedeiro melhor descrito é a captação de hemoglobina por *C. albicans*. Já foi demonstrada atividade hemolítica para este fungo (MANNS; MOSSER; BUCKLEY, 1994), o que liberaria a hemoglobina de dentro das hemácias do hospedeiro e esta proteína seria reconhecida na superfície do fungo por uma família de receptores de membrana (WEISSMAN; KORNITZER, 2004). Das cinco proteínas já descritas pertencentes a essa família, todas contêm domínio CFEM (caracterizado por uma sequência de oito resíduos de cisteína espaçados) e apenas uma não é ancorada por glicosilfosfatidilinositol (GPI) (ALMEIDA; WILSON; HUBE, 2009). Após a ligação da hemoglobina nos receptores de membrana, esse complexo seria internalizado em vesículas endocíticas (WEISSMAN et al., 2008). Dentro do vacúolo, o grupo heme seria degradado por uma heme oxigenase, *Ca*Hmx1, em α-biliverdina (PENDRAK et al., 2004), o que possivelmente liberaria o Fe^{2+} para ser utilizado pelo fungo.

A hemoglobina parece representar uma importante fonte de ferro do hospedeiro também para *C. neoformans*, devido ao robusto crescimento do fungo na presença do grupo heme como única fonte de ferro (JUNG et al., 2008). Recentemente foi sugerido que a proteína Cig1 está presente na superfície de *C. neoformans* participando da captação do grupo heme (CADIEUX et al., 2013). Cig1 possivelmente atua como um hemóforo, proteína extracelular de ligação ao grupo

heme, facilitando o acesso a essa molécula (KRONSTAD; CADIEUX; JUNG, 2013). Após a ligação do grupo heme a Cig1, a via de endocítica seria ativada, permitindo a internalização dessa fonte de ferro do hospedeiro (HU et al., 2013), como sugerido para *C. albicans* (WEISSMAN et al., 2008).

As informações sobre os mecanismos de captação de ferro por fungos patogênicos vêm se acumulando rapidamente. O estudo desses mecanismos vem revelando conexões entre moléculas da superfície dos fungos e a aquisição de fontes de ferro do hospedeiro vertebrado (KRONSTAD; CADIEUX; JUNG, 2013). Porém ainda não se sabe sobre as fontes de ferro preferenciais de *Paracoccidioides* spp. e quais moléculas de superfície o fungo pode utilizar durante os mecanismos de captação desse micronutriente no hospedeiro. O que já se sabe é que a disponibilidade de ferro dentro de monócitos é requerida para a sobrevivência de *Paracoccidioides* sp. neste ambiente, uma vez que o efeito da cloroquina sobre o fungo é revertido por FeNTA. A cloroquina é uma droga com propriedade básica, que interfere com o metabolismo celular de ferro, dependente de um pH ácido, diminuindo a disponibilidade desse micronutriente para a sobrevivência de *Paracoccidioides* sp. E FeNTA é um composto de ferro solúvel em pH neutro a alcalino, que seria utilizado pelo fungo, revertendo o efeito da cloroquina (DIAS-MELICIO et al., 2006). Além disso, já foi demonstrado que genes envolvidos com a captação de ferro em *Paracoccidioides* sp. não são regulados positivamente durante a incubação do fungo com sangue humano, sugerindo que essa condição não é ferro limitante para o fungo (BAILÃO et al., 2006), o que sugere que *Paracoccidioides* sp. possa utilizar hemoglobina como fonte de ferro.

II. ARTIGO SUBMETIDO PARA A REVISTA PLOS NEGLECTED TROPICAL DISEASES

PLOS Neglected Tropical Diseases

HEMOGLOBIN UPTAKE BY Paracoccidioides spp. IS RECEPTOR-MEDIATED

--Manuscript Draft--

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Abstract:	Iron is essential for the proliferation of fungal pathogens during infection. The availability of iron is limited due to its association with host proteins. Fungal pathogens have evolved different mechanisms to acquire iron from host; however, little is known regarding how Paracoccidioides species incorporate and metabolize this ion. In this work, iron sources of the host that are used by Paracoccidioides spp. were investigated. Robust fungal growth in the presence of the iron-containing molecules hemin and hemoglobin was observed. Paracoccidioides spp. presented hemolytic activity and the ability to internalize a protoporphyrin ring. Using real-time PCR and nanoUPLC-MSE proteomic approaches, fungal growth in the presence of hemoglobin was shown to result in the positive regulation of transcripts that encode putative hemoglobin receptors, in association with the induction of proteins that are required for amino acid metabolism and vacuolar protein degradation. In fact, one hemoglobin receptor ortholog, Rbt5, was identified as a surface GPI-anchored protein that recognized hemin, protoporphyrin and hemoglobin in vitro. Antisense RNA technology and Agrobacterium tumefaciens-mediated transformation were used in this work to

	generate mitotically stable Pbrbt5 mutants. The knockdown strain presented diminished survival inside macrophages and in mouse spleen when compared with the parental strain, which suggested that Rbt5 could act as a virulence regulator that facilitates the host iron acquisition by Paracoccidioides sp. In summary, our data indicate that Paracoccidioides spp. can use hemoglobin as an iron source most likely through receptor-mediated pathways that might be relevant for pathogenic mechanisms.
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To

Peter Hotez
Editor-in-Chief
PLOS Neglected Tropical Diseases

Goiânia, December 16th, 2013.

It is our pleasure to submit our article entitled "Hemoglobin uptake by *Paracoccidioides* spp. is receptor-mediated" to the *PLOS Neglected Tropical Diseases* editorial board.

This manuscript has been submitted to PLOS Pathogens in December 9th (manuscript number: PPATHOGENS-D-13-03181). According to the editor Aaron Mitchell, specifically, they had a panel of six editors who read the manuscript and discussed it. The next comments they sent to us: "We were in agreement that the study addresses an important question, and the data are extensive and solid". They were concerned that the main findings are confirmatory of what is known in other fungal pathogens, and thus the novelty of the conclusions is limited. "Several editors suggested that PLOS Neglected Tropical Diseases might be a good journal for this study, since *Paracoccidioides* clear fits into their portfolio. The journal staff may be able to assist you in transferring your manuscript files. Please contact them at plospathogens@plos.org if you wish to pursue that option."

The genus *Paracoccidioides* comprises human thermal dimorphic fungi, causing paracoccidioidomycosis (PCM), an important mycosis in Latin America. This manuscript focuses in this dimorphic fungus, which causes a disease that commonly presents a respiratory chronic manifestation and eventually disseminated lesions. The disease is

considered the most prevalent systemic fungal infection in Brazil and is present in Latin American countries from Mexico to Argentina.

Adaptation to environmental conditions is key to fungal survival during infection of human hosts. Iron is essential for proliferation of fungal pathogens during infection. The availability of iron is limited due to its association to host proteins. Fungal pathogens have evolved different mechanisms to acquire iron from host, but little is known about how *Paracoccidioides* species incorporate and metabolize this ion.

Studies had demonstrated that iron availability is required for *Paracoccidioides* spp. survival. In this way, host's iron sources used by *Paracoccidioides* spp. were investigated in this work. Robust fungal growth in presence of hemin and hemoglobin was observed. *Paracoccidioides* spp. presented hemolytic activity and ability to internalize a protoporphyrin ring. Using real time PCR and nanoUPLC-MS^E proteomic approaches, it was demonstrated that fungal growth in presence of hemoglobin resulted in positive regulation of transcripts encoding predictable hemoglobin receptors, in association with the induction of proteins required for amino acid metabolism and vacuolar protein degradation. In fact, we identified a hemoglobin receptor orthologue, Rbt5, a surface, GPI-anchored protein that recognized hemin, protoporphyrin and hemoglobin *in vitro*.

Functional genomic studies in *Paracoccidioides* spp. have been relatively recent because little is known regarding homologous recombination in this fungus and because the *Paracoccidioides* spp. sexual stage has not been well characterized. This paucity of data compromises the development of efficient classical genetic techniques. Thus, antisense RNA technology and *Agrobacterium tumefaciens*-mediated transformation were used to generate mitotically stable *Pbrbt5* mutants. The knockdown strain presented diminished survival inside macrophages and in mouse spleen when compared with the parental strain, suggesting that Rbt5 could participate in the establishment of *Paracoccidioides* spp. infection. In summary, our data indicate that *Paracoccidioides* spp. can use hemoglobin as an iron source probably through a receptor-mediated pathway that might be relevant for pathogenic mechanisms.

We strongly believe that this study is relevant to further characterizations of the genus *Paracoccidioides* pathobiology, still considered an agent of a neglected disease,

since systems for iron uptake play a major role in adaptation to host niches and are essential factors for the fungal survival and virulence in the mammalian host.

This article is part of our work on identification and characterization of molecules associated to the fungus host interaction as we had described in some recent publications. We believe that this article represents a great contribution for those involved in this area.

We hope to hear from you soon

Sincerely,

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HEMOGLOBIN UPTAKE BY *Paracoccidioides* spp. IS RECEPTOR-MEDIATED

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Keywords: iron physiology; Rbt5; proteome; antisense RNA technology; *Agrobacterium tumefaciens*-mediated transformation.

Abstract

Iron is essential for the proliferation of fungal pathogens during infection. The availability of iron is limited due to its association with host proteins. Fungal pathogens have evolved different mechanisms to acquire iron from host; however, little is known regarding how *Paracoccidioides* species incorporate and metabolize this ion. In this work, iron sources of the host that are used by *Paracoccidioides* spp. were investigated. Robust fungal growth in the presence of the iron-containing molecules hemin and hemoglobin was observed. *Paracoccidioides* spp. presented hemolytic activity and the ability to internalize a protoporphyrin ring. Using real-time PCR and nanoUPLC-MS^E proteomic approaches, fungal growth in the presence of hemoglobin was shown to result in the positive regulation of transcripts that encode putative hemoglobin receptors, in association with the induction of proteins that are required for amino acid metabolism and vacuolar protein degradation. In fact, one hemoglobin receptor ortholog, Rbt5, was identified as a surface GPI-anchored protein that recognized hemin, protoporphyrin and hemoglobin *in vitro*. Antisense RNA technology and *Agrobacterium tumefaciens*-mediated transformation were used in this work to generate mitotically stable *Pbrbt5* mutants. The knockdown strain presented diminished survival inside macrophages and in mouse spleen when compared with the parental strain, which suggested that Rbt5 could act as a virulence regulator that facilitates the host iron acquisition by *Paracoccidioides* sp. In summary, our data indicate that *Paracoccidioides* spp. can use hemoglobin as an iron source most likely through receptor-mediated pathways that might be relevant for pathogenic mechanisms.

Author summary

Fungal infections contribute substantially to human morbidity and mortality. During infectious processes, fungi have evolved mechanisms to obtain iron from high-affinity iron-binding proteins. In the current study, we demonstrated that hemoglobin is the preferential host iron source for the thermodimorphic fungus *Paracoccidioides* spp. To acquire hemoglobin, the fungus presents hemolytic activity and the ability to internalize protoporphyrin rings. A putative hemoglobin receptor, Rbt5, was demonstrated to be GPI-anchored at the yeast cell surface. Rbt5 was able to bind to hemin, protoporphyrin and hemoglobin *in vitro*. When *rbt5* expression was inhibited,

the survival of *Paracoccidioides* sp. inside macrophages and the fungal burden in mouse spleen diminished, which indicated that Rbt5 could participate in the establishment of the fungus inside the host. Drugs or vaccines could be developed against *Paracoccidioides* sp. Rbt5 to disturb iron uptake of this micronutrient and, thus, the proliferation of the fungus. Moreover, this protein could be used in routes to introduce antifungal agents into fungal cells.

Introduction

Iron is an essential micronutrient for almost all organisms, including fungi. Because iron is a transition element, iron can participate as a cofactor in a series of biological processes, such as respiration and amino acid metabolism, as well as DNA and sterol biosynthesis [1]. However, at high levels, iron can be toxic, generating reactive oxygen species (ROS). The regulation of iron acquisition in fungi is one of the most critical steps in maintaining iron homeostasis because this regulation has not been described as possessing a regulated mechanism of iron egress [2].

Although microbial iron acquisition is an important virulence mechanism, the mammal host actively regulates intracellular and systemic iron levels as a mechanism to contain microbial infection and persistence. One strategy to protect the body against iron-dependent ROS cascades and to keep iron away from microorganisms is to tightly bind the metal to many proteins, including hemoglobin, ferritin, transferrin and lactoferrin [3]. In human blood, 66% of the total circulating body iron is bound to hemoglobin. Each hemoglobin molecule possesses four heme groups, and each heme group contains one ferrous ion (Fe^{2+}) [4]. Iron that is bound to the glycoprotein transferrin, which presents two ferric ion (Fe^{3+}) high affinity binding sites, circulates in mammalian plasma [5]. Lactoferrin is present in body fluids, such as serum, milk, saliva and tears [6]. Additionally, similar to transferrin, lactoferrin possesses two Fe^{3+} binding sites [7]. Lactoferrin functions as a defense molecule due to its ability to sequester iron [8]. Although these proteins are important in sequestering extracellular iron, ferritin is primarily an intracellular iron storage protein [9] and is composed of 24 subunits that are composed of approximately 4500 Fe^{3+} ions [10].

Most microorganisms can acquire iron from the host by utilizing high-affinity iron-binding proteins. Preferences for specific host iron sources and strategies to gain iron that is linked to host proteins are under study. It has been revealed, for example, that *Staphylococcus aureus* preferentially uses iron from heme rather than from transferrin during early infection [11]. However, thus far, there is a scarcity of data from pathogenic fungi. It has been suggested that *Cryptococcus neoformans* preferentially uses transferrin as the host iron source through a reductive iron uptake system because Cft1 (*Cryptococcus* Fe Transporter) is required for transferrin utilization and is essential for full virulence [12]. *Histoplasma capsulatum* seems to preferentially use transferrin as the host iron source but also uses hemin and ferritin [13,14]. *Candida albicans* can also mediate iron acquisition from transferrin [15]. Moreover, the Als3 (Agglutinin-like sequence) protein functions as a receptor at the surface of *C. albicans* hyphae, which could support iron acquisition from ferritin [16].

The strategy for iron acquisition from hemoglobin by *C. albicans* is the best characterized. *C. albicans* presents hemolytic activity and utilizes hemin and hemoglobin as iron sources [17-20]. For erythrocyte lyses, *C. albicans* most likely possesses a hemolytic factor that is attached to the fungal cell surface [21]. After hemoglobin release, surface receptors, e.g., Rbt5 (Repressed by Tup1), Rbt51, Wap1/Csa1 (*Candida* Surface Antigen), Csa2 and Pga7 (Predicted GPI-Anchored), could function in the uptake of hemoglobin [19]. Those receptors possess a CFEM domain, which is characterized by a sequence of eight spaced cysteine residues [22]. It has been demonstrated that *rbt5* and *wap1* are transcriptionally activated during low iron conditions (10 μM) in comparison with high iron conditions (100 μM), which indicates that these encoding proteins are important in high-affinity iron uptake pathways [23]. Rbt5, which is a glycosylphosphatidylinositol (GPI)-anchored protein, appears to have a central role in hemin/hemoglobin uptake because the *rbt5* deletion impaired *C. albicans* growth in the presence of hemin and hemoglobin as iron sources [19]. However, *rbt5* deletion did not affect *C. albicans* virulence in a mouse model of systemic infection or during rabbit corneal infection [24], which indicates that other compensatory mechanisms could act in the absence of Rbt5 [19]. It is suggested that after hemoglobin binds to Rbt5, the host iron source is internalized by endocytosis into vacuoles [20].

Recently, it has been proposed that the *C. neoformans* mannoprotein cytokine-inducing glycoprotein (Cig1) acts as a hemophore at the cell surface, which sequesters heme for internalization via a receptor that has not yet been described [25]. After heme binding, the molecule is most likely internalized via endocytosis with the participation of the ESCRT pathway [26], as described for *C. albicans* [20]. In *C. neoformans*, the deletion of *vps23*, which is an ESCRT-I component, resulted in a growth defect on heme and reduced susceptibility to non-iron metalloporphyrins, which have heme-uptake dependent toxicity, indicating that the endocytosis pathway is important for hemoglobin utilization by this fungus [26].

In the host, macrophages play an important role in maintaining adequate levels of plasma iron. Those cells phagocyte aged or damaged erythrocytes and internally recycle iron from senescent erythrocytes [5]. Macrophages are the first host defense cells that interact with *Paracoccidioides* spp. [27], which is a complex of two suggested species (*P. brasiliensis* and *P. lutzii*) of thermodimorphic fungi [28]. Here, this complex is designated as *Paracoccidioides*. All strains of *Paracoccidioides* that have been described thus far are causative agents of paracoccidioidomycosis (PCM) [28], which is a systemic mycosis [29]. Non-activated macrophages are permissive to intracellular *Paracoccidioides* multiplication, functioning as a protected environment against complement systems, antibodies and innate immune components and thus leading to fungal dissemination from the lungs to other tissues [30,31]. Possible strategies that are used by *Paracoccidioides* to survive inside macrophages include (i) the downregulation of macrophage genes that are involved in the inflammatory response and in the activation against pathogens [32,33], (ii) the inhibition of phagosome-endosome fusion [34] and (iii) the detoxification of ROS that are produced by the phagocyte NADPH oxidase system [35]. Moreover, iron availability inside monocytes is required for *Paracoccidioides* survival because the effect of chloroquine on fungal survival is reversed by FeNTA, which is an iron compound that is soluble in the neutral to alkaline pH range [36].

The host iron sources that are used by *Paracoccidioides* have not been established to date. In this work, we demonstrate that *Paracoccidioides* can use hemoglobin as an iron source through a receptor-mediated pathway during infection. This observation unravels new mechanisms by which *Paracoccidioides* species might interfere with the physiology of host tissues.

Results

Hemoglobin and heme group uptake by *Paracoccidioides*

The *Paracoccidioides* strains *Pb*01 and *Pb*18 were grown in the absence of iron (by adding 50 μM BPS, an iron chelator), or in the presence of different iron sources, after 36 h of iron scarcity to deplete intracellular iron storage **(Figure 1)**. The host iron sources that were tested in this work included hemoglobin, ferritin, transferrin and lactoferrin. An inorganic iron source, ferrous ammonium sulfate, was also used. In all conditions, 50 μM BPS was added to verify that the chelator itself does not interfere with *Paracoccidioides* growth. Although some subtle differences were observed in the growth profiles, *Pb*01 and *Pb*18 were able to grow efficiently in the presence of different host iron sources, primarily hemoglobin and ferritin for both strains, and transferrin primarily for the *Pb*01 strain. In iron-depleted medium, *Paracoccidioides* grew poorly. Notably, both *Pb*01 and *Pb*18 presented a robust growth in the presence of hemoglobin or hemin as sole iron sources **(Figure 1)**, which suggested that the increased growth in the presence of hemoglobin was not only due to the amino acid portion but also due to the heme group. These results indicate that hemoglobin could represent an important iron source for *Paracoccidioides* in the host environment.

The robust growth in the presence of hemoglobin and hemin led us to investigate the ability of *Paracoccidioides* to internalize protoporphyrin rings. For this assay, the fungus was incubated for 2 hours in the presence or absence of 20 μM zinc-protoporphyrin IX (Zn-PPIX). The protoporphyrin ring is intrinsically fluorescent, but iron is an efficient quencher of this fluorescence. Consequently, the heme group is not fluorescent, but Zn-PPIX is [37]. Both *Pb*01 and *Pb*18 presented the ability to internalize the protoporphyrin ring because the fluorescence was observed only in fungi that were cultivated with Zn-PPIX **(Figure 2)**. This observation suggests that, to acquire iron from heme, *Paracoccidioides* may internalize the entire molecule to release the iron intracellularly, instead of promoting the iron extraction outside before taking this ion up into cells.

To use hemoglobin as an effective iron source, microorganisms can lyse host erythrocytes to expose the intracellular hemoglobin. The hemolytic ability of

Paracoccidioides was assessed by incubating the fungus for 2 hours after iron starvation with sheep erythrocytes. Both *Pb*01 and *Pb*18 demonstrated the ability to lyse erythrocytes compared with phosphate buffered saline solution (PBS), which was used as a negative control **(Figure 3)**. Sterile water was used as a positive control. This result suggests that *Paracoccidioides* produces a hemolytic factor that can be secreted or that is associated with the fungus surface.

Transcriptional analysis of *Paracoccidioides* genes that encode putative receptors for hemoglobin uptake

In silico searches in the *Paracoccidioides* genome database (http://www.broadinstitute.org/annotation/genome/paracoccidioides_brasiliensis/MultiHome.html) were performed to verify whether the *Pb*01 and *Pb*18 genomes contain genes that encode hemoglobin receptors that are orthologous to genes in the *C. albicans* hemoglobin receptor gene family [19]. The *Pb*01 genome presents three putative hemoglobin receptors that are orthologous to *C. albicans* Rbt5, Wap1/Csa1 and Csa2, respectively. However, *Pb*18 presents only one ortholog to *C. albicans* Wap1/Csa1. Additionally, *Pb*03, which is the other *Paracoccidioides* strain that has its genome published, presents two orthologs, one to *C. albicans* Rbt51 and the other one to *C. albicans* Csa2, as suggested by the *in silico* analysis **(Table S1)**. All four putative identified proteins are predicted to have a CFEM domain, which presents eight spaced cysteine residues [22]. These *in silico* analyses suggest that *Paracoccidioides* could uptake hemoglobin through a receptor-mediated process.

To analyze the expression of *Pb*01 genes that encode putative hemoglobin receptors, real-time qRT-PCRs were performed with the transcripts that encode the *Pb*01 proteins Rbt5, Wap1/Csa1 and Csa2 in different iron supplementation conditions. As depicted in **Figure 4**, the expression of *Pb*01 putative hemoglobin receptors were regulated at the transcriptional level. *Pb01 rbt5* was strongly activated after 120 min in the presence of 10 μM inorganic iron or 10 μM hemoglobin, in comparison with the no iron addition condition. This result suggests that *Pb*01 Rbt5 may participate in an iron acquisition pathway that is involved in hemoglobin iron uptake. The *Pb*01 *csa2* transcript presented the same profile, which suggests that *Pb*01 Csa2 may also work in hemoglobin binding/uptake. The *Pb*wap1/csa1 transcript was activated after 30 min of

incubation in the presence of 10 μM hemoglobin, which suggests that *Pb*01 Wap1/Csa1 might be involved in earlier stages of hemoglobin acquisition.

Paracoccidioides proteins were induced or repressed in the presence of hemoglobin

A nanoUPLC-MS^E-based proteomics approach was employed to identify *Pb*01 proteins that were induced or repressed in the presence of 10 μM hemoglobin as the iron source compared with 10 μM ferrous ammonium sulfate, which was used as an inorganic iron source. In total, 282 proteins were positively or negatively regulated. In this group, 159 proteins were induced **(Table S2)** and 123 proteins were repressed **(Table S3)** in the presence of hemoglobin, compared with proteins that were produced in the presence of inorganic iron. The false positive rates of protein identification in the presence of hemoglobin and in the presence of inorganic iron were 0.58% and 0.30%, respectively. In total, 75.42% and 76.87% of the peptides that were obtained in the presence of hemoglobin and in the presence of inorganic iron, respectively, were identified in a 5 ppm error range **(Figure S1)**. The resulting peptide data that were generated by the PLGS process are shown in **Figure S2**. Some proteins that were induced or repressed in the presence of hemoglobin are depicted in **Tables 1 and 2**, respectively. Many proteins that were detected are involved in amino acid, nitrogen and sulfur metabolism **(Tables 1 and 2)**. Proteins that are involved in alanine, lysine, tryptophan or aspartate and glutamate metabolism, as well as those proteins that are involved in arginine, cysteine, histidine, serine or threonine biosynthesis, were upregulated in the presence of hemoglobin **(Table 1)**. In contrast, proteins that are involved in asparagine or phenylalanine degradation were down regulated in the presence of hemoglobin **(Table 2)**. This result suggests that the fungus could use hemoglobin not only as iron source, as demonstrated by the induction of proteins that are involved with the iron-sulfur cluster assembly, such as cysteine desulfurase **(Table 1)**, but also as nitrogen and sulfur sources because many proteins that are involved in amino acid metabolism were upregulated in the presence of hemoglobin. This observation reinforces the notion that *Paracoccidioides* internalizes the entire hemoglobin molecule instead of promoting the iron release extracellularly. This internalization could occur by endocytosis because proteins that are involved with lysosomal and vacuolar protein degradation, including carboxypeptidase Y and a vacuolar protease A ortholog, were upregulated **(Table 1)**. Among the induced proteins, it is important to highlight the *Pb*01 Csa2 detection only in presence of hemoglobin

(Table 1), which corroborates the hypothesis that hemoglobin uptake by *Paracoccidioides* is receptor-mediated. Among the repressed proteins, those proteins that are involved with porphyrin biosynthesis, including uroporphyrinogen decarboxylase and a glutamate-1-semialdehyde 2,1-aminomutase ortholog, were detected only in the presence of inorganic iron **(Table 2)**, which reinforces the hypothesis that hemoglobin is efficiently used by the fungus.

Paracoccidioides Rbt5 is a GPI-anchored cell wall protein

We continued our studies with the *Pb*01 ortholog of Rbt5, the best-characterized hemoglobin receptor in *C. albicans* [20]. As described above, *Pb*01 *rbt5* was also the transcript that was most efficiently regulated in the presence of hemoglobin. To investigate this result further, a recombinant GST-tagged *Pb*01 Rbt5 protein, which was 42.5 kDa, was produced in *Escherichia coli* and purified using the GST tag. After purification, the GST tag was removed after thrombin digestion, and the resultant protein presented a molecular mass of 22 kDa **(Figure S3A)**. Polyclonal antibodies were raised against the recombinant protein in rabbit. To verify the reactivity of the obtained antibody against the recombinant protein, Western blots were performed **(Figures S3B and S3C)**. Only a 22 kDa immunoreactive species was obtained in the Western blot analysis after GST tag cleavage **(Figure S3B, lane 3)**.

In silico analysis identified a predicted signal peptide and a putative GPI anchor in the *Pb*01 Rbt5 ortholog, which was similar to *C. albicans* Rbt5 **(Figure S4)**, suggesting that this protein could localize at the *Paracoccidioides* cell surface. In this way, the GPI-anchored proteins of the *Pb*01 cell wall were extracted using HF-pyridine. A Western blot assay was performed using anti-*Pb*01 Rbt5 polyclonal antibodies against the GPI-anchored protein extract, and a single immunoreactive 60 kDa species was identified in this fraction **(Figure 5A, lane 2)**. The mass shift from 22 kDa to 60 kDa suggests post-translational modifications (PTMs) of the native protein, which is in agreement with the occurrence of glycosylation [38].

To confirm the cell wall localization, an immunocytochemical analysis of *Pb*01 yeast cells using anti-*Pb*01 Rbt5 polyclonal antibodies was prepared for analysis using transmission electron microscopy **(Figure 5B, panels 2 and 3)**. Rbt5 was abundantly detected on the *Pb*01 yeast cell wall. Some gold particles were observed in the cytoplasm, which is consistent with intracellular synthesis for further surface export.

The control sample was free of label when incubated with the rabbit preimmune serum **(Figure 5B, panel 1)**.

Paracoccidioides Rbt5 binds heme-containing molecules

The fact that *Pb*01 Rbt5 is homologous to *C. albicans* Rbt5 **(Figure S4)** suggests that *Pb*01 Rbt5 may participate in hemoglobin uptake in *Paracoccidioides*. In this way, the protein's ability to interact with the heme group was investigated. Affinity assays were performed using the recombinant *Pb*01 Rbt5 and a hemin-agarose resin **(Figure 6A)**. A specific ability to interact with hemin was demonstrated by *Pb*01 Rbt5 **(Figure 6A, lane 1)**. Enolase, which is also present at the *Pb*01 yeast surface [39], was used as a negative control in this assay. As expected, enolase was not able to bind hemin. For this reason, the purified protein was not linked to the hemin-agarose resin **(Figure 6A, lane 2)**.

To confirm the ability of Rbt5 to recognize heme-containing molecules, *Pb*01 yeast cells were submitted to binding assays for further flow cytometry analyses. Background fluorescence levels were determined using yeast cells alone **(Figure 6B, black lines)**. Positive controls were composed of systems where *Pb*01 cells were incubated with polyclonal antibodies raised against *Pb*Rbt5, followed by incubation with a fluorescent secondary antibody **(Figure 6B, red lines)**. For the determination of binding activities, *Pb*01 yeast cells were incubated with protoporphyrin or hemoglobin before or after exposure to the anti-*Pb*Rbt5 antibodies, followed by incubation with the secondary antibody **(Figure 6B, green and blue lines, respectively)**. When yeast cells were incubated with protoporphyrin or hemoglobin before exposure to primary and secondary antibodies, fluorescence intensities were at background levels, suggesting that the heme-containing proteins blocked surface sites that are also recognized by the anti-*Pb*Rbt5 antibodies **(Figure 6B)**. When the cells were exposed to protoporphyrin or to hemoglobin after incubation with the antibodies, the fluorescence levels were similar to those levels that were obtained in systems where incubation with the heme-containing proteins was omitted. These results suggest a high-affinity binding between Rbt5 and heme-containing molecules, which corroborates the hypothesis that Rbt5 could act as a hemoglobin receptor at the fungus cell surface.

Paracoccidioides rbt5 knockdown decreases survival inside the host

To verify whether Rbt5 deficiency could influence the ability of the fungus to acquire heme groups or to survive inside the host, an antisense-RNA (aRNA) strategy was applied **(Figure 7A)**. For this analysis, *Pb*339 was used because the *Agrobacterium tumefaciens*-mediated transformation (ATMT) of this strain has been standardized [40]. The knockdown strategy was demonstrated to be efficient because the quantification of *rbt5* transcripts in two isolates of knockdown strain (*Pbrbt5*-aRNA) was 60% lower than in the wild type strain (*Pb*Wt) **(Figure 7B)**. The strain that was transformed with the empty vector (*Pb*Wt+EV) showed a similar level of *rbt5* transcripts compared with *Pb*Wt **(Figure 7B)**. Because of its higher stability, the *Pbrbt5*-aRNA 1 isolate was selected for the next experiments. The results with *Pb*Wt and *Pb*Wt+EV strains **(Figure 7C)** were similar to those results that are described in **Figure 6B**. In contrast, fluorescence intensities were all at background levels when the *Pb*rbt5-aRNA strain was assessed. These results suggest that Rbt5 in the *Pb*339 strain also has an affinity for hemoglobin, which possibly exerts the function of a heme-binding receptor.

Despite the efficiency of the knockdown strategy, the *Pbrbt5*-aRNA strain demonstrated an identical ability to grow in the presence of hemoglobin as the iron source as than the other strains **(Figure S5)**, which suggests that either a low amount of Rbt5 at the cell surface is sufficient to allow hemoglobin acquisition or that the other putative hemoglobin receptors could compensate for the Rbt5 deficiency. The identical growth ability of all three strains was also observed in media without iron and with ferrous ammonium sulfate as an inorganic iron source **(Figure S5)**.

To test the ability of *Paracoccidioides* mutant strains to survive inside the host, two strategies were employed. First, *Pbrbt5*-aRNA and *Pb*Wt+EV were co-cultivated with macrophages. *Pb*Wt was used as a control. After 24 h, macrophages were first washed with PBS to remove the weakly bounded yeast cells and then were lysed with distilled water. Lysates were plated on BHI solid medium to recover the internalized fungi. After 10 days, the colony forming units (CFUs) were counted, and the *Pbrbt5*-aRNA presented approximately 98% reduction in the number of CFUs in comparison with *Pb*Wt and *Pb*Wt+EV **(Figure 8A)**. The second strategy included a murine model of infection. Mice were inoculated intraperitoneally with *Pb*Wt, *Pb*Wt+EV and *Pbrbt5*-aRNA, independently. After 2 weeks of infection, the mice were sacrificed, and the spleens were removed. The organs were macerated, and the homogenized sample was plated on BHI agar for CFU determination. The number of CFUs after the infection

with the *Pbrbt5*-aRNA strain was approximately 6 times lower than the CFUs that were observed after the infection with *Pb*Wt or with *Pb*Wt+EV **(Figure 8B)**. These results indicate that the *rbt5* knockdown could reduce the virulence of the fungi and/or increase the stimulation of the host defense cells to kill the fungus.

To verify whether *Pb*Rbt5 had antigenic properties, sera of five PCM patients were used in immunoblot assays against the recombinant protein. All sera presented strong reactivity against the recombinant *Pb*01 Rbt5 that was immobilized in the nitrocellulose membrane **(Figure 8C, lanes 1-5)**. No cross-reactivity was observed with control sera of patients who were not diagnosed with PCM **(Figure 8C, lanes 6-10)**. This result suggests that *Pb*01 Rbt5 is an antigenic protein that is produced by *Paracoccidioides* during human infection.

Discussion

Because pathogenic fungi face iron deprivation in the host, these microorganisms have evolved different mechanisms to acquire iron from the host's iron-binding proteins [41]. *C. albicans*, for example, can use transferrin, ferritin and hemoglobin as host iron sources [15,16,19,20]. It has been demonstrated in *Paracoccidioides* that genes that are involved in iron acquisition are not upregulated during the incubation of the fungus with human blood, which suggests that this condition is not iron-limiting for this fungus [42]. This observation, coupled with the *Paracoccidioides* preference for heme iron in culture, suggests heme iron scavenging during infection.

In this study, we observed that *Paracoccidioides* presented the ability to internalize a zinc-bound protoporphyrin ring. Moreover, the fungus could promote erythrocyte lysis. A hemolysin-like protein (XP_002797334) has been evidenced in a mycelium to yeast transition cDNA library [43], which indicates that *Paracoccidioides* could access the intracellular heme in the host by producing a hemolytic factor that can be secreted or associated with the fungus surface. The ability to internalize the zinc-bound protoporphyrin ring has been demonstrated for *C. albicans*, but not for *C. glabrata* [37]. The absence of protoporphyrin internalization by *C. glabrata* is most likely because heme receptors are not present in this fungus, as suggested by the fact

that genes that encode these receptors have not been identified in the *C. glabrata* genome [37]. In contrast, a hemoglobin-receptor gene family that is composed of the genes *rbt5*, *rbt51*, *wap1/csa1*, *csa2* and *pga7* has been identified in *C. albicans* [19]. To access the heme group inside the erythrocytes, *C. albicans* also produces a hemolytic factor that is able to promote the lysis of erythrocytes [17].

By performing an *in silico* analysis, iron-related genes were identified in the *Paracoccidioides* genome, which were composed of *rbt5*, *wap1/csa1* and *csa2*, that were orthologous to *C. albicans* genes that encode hemoglobin receptors [19], providing further evidence that *Paracoccidioides* has the ability to use hemoglobin in its regular metabolic pathways. In *C. albicans*, the transcripts *rbt5* and *wap1* are activated during low iron condition compared with high iron abundance conditions [23], which corroborates the hypothesis that these transcripts are involved in an iron acquisition mechanism, more specifically, in hemoglobin uptake. Similar results were obtained with *Paracoccidioides*. The *rbt5*, *wap1/csa1* and *csa2* transcripts were also induced when the fungus *Pb*01 was cultivated in a low iron condition compared with high iron abundance condition. Moreover, these transcripts were induced in a low-inorganic iron condition or in the presence of hemoglobin compared with the fungus growth in conditions with no addition of iron. These results suggest that the proteins that are encoded by the analyzed transcripts could be involved in hemoglobin utilization in *Pb*01.

The proteomic analysis of the *Pb*01 strain demonstrated that Csa2 was detected only in the presence of hemoglobin, which suggests that its uptake by *Paracoccidioides* is receptor-mediated, as described for *C. albicans* [19] and *C. neoformans* [25]. Among the three *Pb*01 hemoglobin-receptor orthologs, Csa2 is the only one that was not predicted to have a GPI-anchor **(Table S1)**. Because no specific protocol to purify GPI-surface proteins has been used for proteomic analyses, no additional hemoglobin-binding proteins than Csa2 were identified in this proteome. The *Pb*01 proteome in the presence of hemoglobin demonstrated that proteins that are involved in amino acid, nitrogen and sulfur metabolism and in iron-sulfur cluster assembly were induced in comparison with the fungus that was cultivated in presence of inorganic iron. Moreover, proteins that are involved in porphyrin biosynthesis were detected only when the fungus was cultivated in the presence of inorganic iron. These results suggest that the fungus could use hemoglobin as an efficient source of nitrogen, sulfur, iron and porphyrin, internalizing the entire hemoglobin molecule. This internalization hypothesis is

corroborated by the fact that proteins that are involved with lysosomal and vacuolar protein degradation were also induced in the presence of hemoglobin. Similar mechanisms have been suggested for *C. albicans* and *C. neoformans*. In *C. albicans*, hemoglobin is taken up by endocytosis after Rbt5/51 binding [20]. In *C. neoformans*, Cig1, a recently described extracellular mannoprotein that functions as a receptor or hemophore at the cell surface [25] potentially helps the fungus to take up heme before iron release, perhaps by endocytosis [26].

*Pb*01 *rbt5* presented a high level of transcriptional regulation in the presence of hemoglobin, as observed in this work. In this way, *Pb*01 Rbt5 was investigated and demonstrated characteristics that were similar to *C. albicans* Rbt5, such as the presence of a CFEM domain [9] and a GPI anchor [44]. *Pb*01 Rbt5 was identified in the cell wall extract, which was enriched with GPI proteins that was obtained as previously described [45], and was visualized at the *Pb*01 yeast surface. These results indicate that *Pb*01 Rbt5 is anchored at the fungal surface through a GPI anchor. To function as a hemoglobin receptor, a protein must be able to bind heme. *Pb*01 Rbt5 heme group-binding ability was demonstrated using batch ligand affinity chromatography, with a hemin-resin and the *Pb*01 Rbt5 recombinant protein. Moreover, flow cytometry assays using the whole *Pb*01 yeast cells and the anti-Rbt5 antibodies, which were raised against the *Pb*01 Rbt5 recombinant protein, in the presence of protoporphyrin and hemoglobin also demonstrated the *Pb*01 Rbt5 affinity for these two heme-containing molecules. In *C. neoformans*, the Cig1 heme-binding ability was detected using spectrophotometric titration and isothermal titration calorimetry (ITC) assays with recombinant Cig1-GST protein purified from *E. coli* [25]. These results demonstrated that *Pb*01 Rbt5 is able to bind hemin, protoporphyrin and hemoglobin, which corroborates the hypothesis that *Pb*01 Rbt5 could function as a heme group receptor, which could help in the acquisition of iron from host sources.

Functional genomic studies in *Paracoccidioides* are recent because little is known regarding the fungi's life cycle. For instance, mechanisms of homologous recombination or haploid segregation in *Paracoccidioides* cells remain obscure. This paucity of data compromises the development of efficient classical genetic techniques [46]. Thus, to modulate the expression of target genes in *Paracoccidioides*, antisense RNA (aRNA) technology is applicable by *A. tumefaciens*-mediated transformation (ATMT) [40,46,47]. In this work, *Paracoccidioides rbt5* knockdown strains were

generated using the same methodologies. Reductions in gene and protein expression in *Pbrbt5*-aRNA strains were demonstrated by qRT-PCR and flow cytometry assays, respectively, in comparison with the control strains (wild type and empty vector transformed strains). Despite the knockdown of *Pb*Rbt5, no growth difference was observed in the presence of inorganic iron or hemoglobin sources. In contrast, in *C. albicans*, a *Δrbt5* mutant strain presented reduced growth in the presence of hemin and hemoglobin as iron sources [19]. These results suggest that other hemoglobin receptors could function at the *Paracoccidioides* surface; this possibility is the focus of future studies in our laboratory.

Paracoccidioides is a thermodimorphic fungus that can infect the host by airborne propagules. After the mycelium-yeast transition in the host lungs, the fungus can disseminate to different organs and tissues through the hematogenous or lymphatic pathways [48,49]. In the host tissues, including the lungs, the fungus can be internalized by macrophages [30,34]. One of the functions of the macrophages is to recycle senescent red cell iron, primarily in the spleen. Hemoglobin-derived heme is catabolized and the heme iron is released by a hemoxygenase inside macrophages [5]. In this way, *Paracoccidioides* has at least two different opportunities to be exposed to the heme group: during (i) fungal dissemination by the hematogenous route or (ii) infection of macrophages. Because it has been suggested that monocyte intracellular iron availability is required for *Paracoccidioides* survival [36], the ability of the *rbt5* knockdown strain to survive inside macrophages was investigated. The *rbt5* knockdown strain presented decreased survival inside macrophages in comparison with control strains, which indicates that Rbt5 could be a virulence factor and/or could affect macrophage stimulation to kill the internalized yeast cells. In addition, the fungal burden in mouse spleen that was infected with the *rbt5* knockdown strain was lower than the fungal burden of the mice that were infected with the control strains, indicating that Rbt5 could be important for infection establishment and/or maintenance by *Paracoccidioides*. In contrast, the *rbt5* deletion did not affect *C. albicans* virulence in animal models of infection [24], which indicates that other compensatory mechanisms could act in the absence of Rbt5 in this fungus [19]. The ability of Rbt5 to function as an antigen in *Paracoccidioides* was demonstrated by *Pb*01 Rbt5 recombinant protein recognition using sera of five PCM patients in immunoblot assays. Similar results were obtained for *C. albicans* because Rbt5 and Csa1 were found among 33 antigens that

were recognized by sera from convalescent candidemia patients [50]. These results reinforce that Rbt5 could act in the host-pathogen interface.

Fungal surface proteins that are involved in iron uptake might be attractive targets for vaccines or drugs that block microbial proliferation. Moreover, these proteins could be considered as routes to introduce antifungal agents into fungal cells [51]. In that way, iron acquisition mechanisms could be important targets to prevent or treat fungal diseases. This study constitutes evidence that *Paracoccidioides* could acquire heme groups through a receptor-mediated mechanism. In that way, Rbt5 may be a good target for developing vaccines, for blocking *Paracoccidioides* proliferation inside phagocytes, or for using a Trojan horse strategy for introducing antifungal agents into yeast cells.

Materials and Methods

Ethics statement

All animals were treated in accordance with the guidelines provided by the Ethics Committee on Animal Use from Universidade Federal de Goiás based on the International Guiding Principles for Biomedical Research Involving Animals (http://www.cioms.ch/images/stories/CIOMS/guidelines/1985_texts_of_guidelines.htm) , and were approved by this committee (131/2008).

Strains and growth conditions

Paracoccidioides strains *Pb*01 (ATCC MYA-826; *Paracoccidioides lutzii*) [28], *Pb*18 (ATCC 32069; *Paracoccidioides brasiliensis*, phylogenetic species S1) and *Pb*339 (ATCC 200273; *Paracoccidioides brasiliensis*, phylogenetic species S1) [52] were used in this work. The fungus was maintained in brain heart infusion (BHI) medium, which was supplemented with 4% (w/v) glucose at 36 °C to cultivate the yeast form. For growth assays, *Paracoccidioides* yeast cells were incubated in chemically defined MMcM medium [53] with no iron addition for 36 h at 36 °C under rotation to deplete intracellular iron reserves. Cells were collected and washed twice with phosphate buffered saline solution 1X (1X PBS; 1.4 mM KH_2PO_4, 8 mM Na_2HPO_4, 140 mM NaCl, 2.7 mM KCl; pH 7.3). Cell suspensions were serially diluted and spotted on

plates with MMcM medium, which contained 50 µM of bathophenanthroline disulfonic acid (BPS) that was supplemented or not (no iron condition) with different iron sources: 30 µM inorganic iron [$Fe(NH_4)_2(SO_4)_2$], 30 µM hemoglobin, 120 µM hemin, 30 µg/ml ferritin, 30 µM transferrin or 3 µM lactoferrin. All host iron sources were purchased from Sigma-Aldrich, St. Louis, MO, USA.

Fluorescence microscopy

Paracoccidioides yeast cells, which were maintained in MMcM medium for 36 h, were incubated on the same medium, which was supplemented or not with 20 µM zinc protoporphyrin IX (zinc-PPIX) (Sigma-Aldrich, St. Louis, MO, USA) for 2 h at 36 °C under rotation. Cells were collected, washed twice with 1X PBS and observed by live fluorescence microscopy using an Axio Scope A1 microscope with a 40x objective and the software AxioVision (Carl Zeiss AG, Germany). The Zeiss filter set 05 was used to detect intrinsic zinc-PPIX fluorescence. The fluorescence background was determined in the absence of zinc-PPIX in the MMcM medium.

Hemolytic activity of *Paracoccidioides*

The hemolytic activity of *Paracoccidioides* was evaluated as described previously [17], with modifications. Briefly, the fungus was cultivated in non-iron addition MMcM medium for 36 h at 36 °C under rotation. After this period, the yeast cells were harvested and washed twice with 1X PBS. Then, 10^7 cells were incubated with 10^8 sheep erythrocytes (Newprov Ltda, Pinhais, Paraná, Brazil) for 2 h, at 36 °C in 5% CO_2. As negative or positive controls, respectively, erythrocytes were incubated with 1X PBS or water. After incubation, the cells were resuspended by gentle pipetting, and then pelleted by brief centrifugation. The optical densities of the supernatants were determined using an ELISA plate reader at 405 nm. The experiment was performed in triplicate, and the average of the optical density was obtained for each condition. The average optical density of each condition was used to calculate the relative hemolysis of the experimental conditions or the negative control against the positive control. The relative hemolysis data were plotted in a bar graph. Student's t-test was applied to compare the experimental values to the negative control values.

***In silico* analysis of *Paracoccidioides* putative hemoglobin receptors**

The amino acid sequences of putative members of the *Paracoccidioides* hemoglobin receptor family were obtained from the Dimorphic Fungal Database of the Broad Institute site at (http://www.broadinstitute.org/annotation/genome/dimorph_collab//MultiHome.html) based on a homology search. The sequences for *Pb*01 Rbt5, Wap1 and Csa2 have been submitted to GenBank with the following respective accession numbers: XP_002793022, XP_002795519 and XP_002797192. For *Pb*18 Wap1, the accession number is EEH49284. And for *Pb*03 Rbt51 and Csa2, the accession numbers are, respectively, EEH22388 and EEH19315. SMART (http://smart.embl-heidelberg.de/), SignalP 4.1 Server (http://www.cbs.dtu.dk/services/SignalP/) and big-PI Fungal Predictor (http://mendel.imp.ac.at/gpi/fungi_server.html) protein analysis tools were used to search for conserved domains, signal peptides and GPI modification sites, respectively, in *Paracoccidioides* and *C. albicans* sequences. The amino acid sequences of *Paracoccidioides* and *C. albicans* orthologs were aligned using the CLUSTALX2 program [54].

RNA extraction and quantitative real time PCR (qRT-PCR)

*Pb*01 yeast cells were incubated in non-iron addition MMcM medium or in MMcM medium that was supplemented with different iron sources: 10 or 100 μM inorganic iron or 10 μM hemoglobin. Cells were harvested after 30, 60 or 120 min of incubation, and total RNA was extracted using TRIzol (TRI Reagent®, Sigma-Aldrich, St. Louis, MO, USA) and mechanical cell rupture (Mini-Beadbeater™ - Biospec Products Inc., Bartlesville, OK). After *in vitro* reverse transcription (SuperScript™ III First-Strand Synthesis SuperMix; Invitrogen™, Life Technologies), the cDNAs were submitted to a qRT-PCR reaction, which was performed using SYBR Green PCR Master Mix (Applied Biosystems, Foster City, CA) in a StepOnePlus™ Real-Time PCR System (Applied Biosystems Inc.). The expression values were calculated using the transcript that encoded *alpha tubulin* (XM_002796593) as the endogenous control as previously reported [55]. The primer pairs for qRT-PCR were designed such that one primer in each pair spanned an intron, which prevented genomic DNA amplification. The sequences of the oligonucleotide primers that were used were as follows: *rbt5*-S, 5'- ATATCCCACCTTGCGCTTTGA -3'; *rbt5*-AS, 5'- GGGCAGCAACGTCGCAAGA -3'; *wap1*-S, 5'- AAGTCTGTGATAGTGCTGGAG -3'; *wap1*-AS, 5'- AGGGGGTTCAGGGAGAGGA -3'; *csa2*-S, 5'-

GCAAAATTAAAGAATCTCTCACG -3'; *csa2*-AS, 5'-ATGAAACGGCAAATCCCACCA-3'; *alpha-tubulin*-S, 5'-ACAGTGCTTGGGAACTATACC -3'; *alpha-tubulin*-AS, 5'-GGGACATATTTGCCACTGCC -3'. The annealing temperature for all primers was 62 °C. The qRT-PCR reaction was performed in triplicate for each cDNA sample, and a melting curve analysis was performed to confirm single PCR products. The relative standard curve was generated using a pool of cDNAs from all the conditions that were used, which was serially diluted 1:5 to 1:625. Relative expression levels of transcripts of interest were calculated using the standard curve method for relative quantification [56]. Student's t-test was applied in the statistical analyses.

Sample preparation, nanoUPLC-MS^E acquisition and protein classification

*Pb*01 yeast cells were cultivated in MMcM medium with 10 μM inorganic iron [$Fe(NH_4)_2(SO_4)_2$] or with 10 μM bovine hemoglobin (H2500-Sigma-Aldrich, St. Louis, MO, USA) at 36 °C under constant agitation. After 48 h, the cells were harvested, and the cell rupture was performed as described above, in the presence of Tris-Ca buffer (Tris-HCl 20 mM, pH 8.8; CaCl2 2 mM) with 1% proteases inhibitor (Protease Inhibitor mix 100x, Amersham). The mixtures were centrifuged at 12,000 g at 4 °C for 10 min. The supernatant was collected and centrifuged again at the same conditions for 20 min. Then, the protein extracts were washed twice with 50 mM NH_4HCO_3 buffer and concentrated using a 10 kDa molecular weight cut-off in an Ultracel regenerated membrane (Amicon Ultra centrifugal filter, Millipore, Bedford, MA, USA). The proteins extracts concentration were determined using the Bradford assay [57]. These extracts were prepared as previously described [58] for analyses using nano-scale ultra-performance liquid chromatography combined with mass spectrometry with data-independent acquisitions (nanoUPLC-MS^E). In this way, the trypsin-digested peptides were separated using a nanoACQUITY^{TM} UPLC System (Waters Corporation, Manchester, UK). The MS data that were obtained via nanoUPLC-MS^E were processed and examined using the ProteinLynx Global Server (PLGS) version 2.5 (Waters Corporation, Manchester, UK). Protein identification and quantification level analyses were performed as described previously [59]. The observed intensity measurements were normalized with the identified peptides of the digested internal standard rabbit phosphorylase. For protein identification, the *Paracoccidioides* genome database was used. Protein tables that were generated by PLGS were merged using the FBAT

software [60], and the dynamic range of the experiment was calculated using the MassPivot software (kindly provided by Dr. Andre M. Murad) by setting the minimum repeat rate for each protein in all replicates to 2 as described previously [59]. Proteins were considered regulated when $p < 0.05$ (determined by PLGS) and when the fold change between protein quantification in the presence of hemoglobin x presence of inorganic iron was $\pm$ 0.2. Proteins were classified according to MIPS functional categorization (http://mips.helmholtz-muenchen.de/proj/funcatDB/) with the help of the online tools UniProt (http://www.uniprot.org/), PEDANT (http://pedant.helmholtz-muenchen.de/pedant3htmlview/pedant3view?Method=analysis&Db=p3_r48325_Par_brasi_Pb01) and KEGG (http://www.genome.jp/kegg/). Graphics that indicated the quality of the proteomic data were generated using the Spotfire software (http://spotfire.tibco.com/).

Expression and purification of recombinant Rbt5

Oligonucleotide primers were designed to amplify the 585 bp complete coding region of Rbt5: *rbt5*-S, 5'- GGTGTCGACCAGCTCCCTAATATCCCAC -3'; *rbt5*-AS, 5'- GGTGCGGCCGCGACATAATTTACAGGTAAGC -3' (underlined regions correspond to *Not*I and *Sal*I restriction sites, respectively). The PCR product was subcloned into the *Not*I/*Sal*I sites of pGEX-4T-3 (GE Healthcare). The DNA was sequenced on both strands and was used to transform the *E. coli* C41 (DE3). The transformed cells were grown at 37 °C, and protein expression was induced by the addition of 1 mM isopropyl β-D- thiogalactopyranoside (IPTG) for 5 h. The bacterial extract was centrifuged at 2,700 g and was resuspended in 1X PBS. The fusion protein Rbt5 was expressed in the soluble form in the heterologous system and was purified by affinity chromatography under non-denaturing conditions using glutathionesepharose 4B resin (GE Healthcare). Subsequently, the fusion protein was cleaved by the addition of thrombin protease (50 U/ml). The purity and size of the recombinant protein were evaluated by resuspending the protein in SDS-loading buffer [50 mM Tris-HCl, pH 6.8; 100 mM dithiothreitol, 2% (w/v) SDS; 0.1% (w/v) bromophenol blue; 10% (v/v) glycerol], followed by boiling for 5 min, and running the purified molecule on a 12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and finally, staining with Coomassie blue.

Antibody production

The purified Rbt5 was used to generate a specific rabbit polyclonal serum. Rabbit preimmune serum was obtained and stored at -20 °C. The purified recombinant protein (300 μg) was injected into rabbit with Freund's adjuvant three times at 10-day intervals. The obtained serum was sampled and stored at -20 °C.

Cell wall protein extractions and enzymatic treatments

Yeast cells were frozen in liquid nitrogen and disrupted by using a mortar and pestle. This procedure was performed until the cells completely ruptured, which was verified by optical microscopic analysis. The ground material was lyophilized, weighed, and resuspended in 25 μl Tris buffer (50 mM Tris-HCl, pH 7.8) for each milligram of dry weight as described previously [61]. The supernatant was separated from the cell wall fraction by centrifugation at 10,000 g for 10 min at 4 °C. To remove proteins that were not covalently linked and intracellular contaminants, the isolated cell wall fraction was washed extensively with 1 M NaCl, was boiled three times in SDS-extraction buffer (50 mM Tris-HCl, pH 7.8, 2% [w/v] SDS, 100 mM Na-EDTA, 40 mM β-mercaptoethanol) and pelleted by centrifugation at 10,000 g for 10 min [62]. The washed pellet containing the cell wall enriched fraction was washed six times with water, lyophilized, and weighed. The cell wall fraction, which was prepared as described above, was treated with hydrofluoric acid-pyridine (HF-pyridine) (10 μl for each milligram of dry weight of cell walls) for 4 h at 0 °C [45,63]. After centrifugation, the supernatant that contained the HF-pyridine extracted proteins was collected, and HF-pyridine was removed by precipitating the supernatant in 9 volumes of methanol buffer (50% v/v methanol, 50 mM Tris-HCl, pH 7.8) at 0 °C for 2 h. The pellet was washed three times in methanol buffer and resuspended in approximately 10 times the pellet volume in SDS-loading buffer, as described previously [63].

Western blotting analysis

Twenty micrograms of protein samples were loaded onto a 12% SDS-PAGE gel and were separated by electrophoresis. Proteins were transferred from gels to nitrocellulose membrane at 30 V for 16 h in buffer that contained 25 mM Tris-HCl pH 8.8, 190 mM glycine and 20% (v/v) methanol. Membranes were stained with Ponceau red to confirm complete protein transfer. Next, each membrane was submerged in blocking buffer [1X PBS, 5% (w/v) non-fat dried milk, 0.1% (v/v) Tween-20] for 2 h. Membranes were washed with wash buffer [1X PBS, 0.1% (v/v) Tween-20] and

incubated with primary antibody, which was used at a 1/3,000 (v/v) ratio of antibody to buffer, for 2 h at room temperature. This step was followed by three 15 min washes with the buffer that was mentioned above. Membranes were incubated with the conjugated secondary antibody [anti-rabbit immunoglobulin G coupled to alkaline phosphatase (Sigma-Aldrich, St. Louis, MO, USA)] in a 1/5,000 (v/v) ratio and developed with 5-bromo-4-chloro-3-indolylphosphate–nitroblue tetrazolium (BCIP-NBT). Reactions were also performed with sera from patients with PCM, sera from control individuals (all diluted 1:100) and with 1 µg of purified recombinant Rbt5. After incubation with peroxidase conjugate anti-human IgG (diluted 1:1000), the reaction was developed with hydrogen peroxide and diaminobenzidine (Sigma-Aldrich, St. Louis, MO, USA) as the chromogenic reagent.

Transmission electron microscopy of *Paracoccidioides* yeast cells and immunocytochemistry of Rbt5

For the ultrastructural and immunocytochemistry studies, the protocols that were previously described by Lima and colleagues [64] were employed. Transmission electron microscopy was performed using thin sections from *Pb*01 yeast that were fixed in 2% (v/v) glutaraldehyde, 2% (w/v) paraformaldehyde and 3% (w/v) sucrose in 0.1 M sodium cacodylate buffer pH 7.2. The samples were post-fixed in a solution that contained 1% (w/v) osmium tetroxide, 0.8% (w/v) potassium ferricyanide and 5 mM $CaCl_2$ in sodium cacodylate buffer, pH 7.2. The material was embedded in Spurr resin (Electron Microscopy Sciences, Washington, PA). Ultrathin sections were stained with 3% (w/v) uranyl acetate and lead citrate. For immunolabeling, the cells were fixed in a mixture that contained 4% (w/v) paraformaldehyde, 0.5% (v/v) glutaraldehyde and 0.2% (w/v) picric acid in 0.1 M sodium cacodylate buffer at pH 7.2 for 24 h at 4 °C. Free aldehyde groups were quenched with 50 mM ammonium chloride for 1 h. Block staining was performed in a solution containing 2% (w/v) uranyl acetate in 15% (v/v) acetone. After dehydration, samples were embedded in LR Gold resin (Electron Microscopy Sciences, Washington, PA.). For ultrastructural immunocytochemistry studies, the ultrathin sections were incubated for 1 h with the polyclonal antibody raised against the recombinant *Pb*01 Rbt5, which was diluted 1:100, and for 1 h at room temperature with the labeled secondary antibody anti-rabbit IgG Au-conjugated (10 nm average particle size; 1:20 dilution; Electron Microscopy Sciences, Washington, PA). The nickel grids were stained as described above and observed using a Jeol 1011

transmission electron microscope (Jeol, Tokyo, Japan). Controls were incubated with a rabbit preimmune serum, which was diluted 1:100, followed by incubation with the labeled secondary antibody.

Hemin-agarose binding assay

The recombinant proteins Rbt5 and Enolase, which were previously obtained in our laboratory [39], were incubated, independently, with a hemin-agarose resin (Sigma-Aldrich, St. Louis, MO, USA) for 1 h at 4 °C. The resin was washed three times with cold 1X PBS, resuspended in SDS-loading buffer and boiled for 5 min to elute proteins that were bound to the resin. The samples were submitted to SDS-PAGE, and the proteins were transferred to nitrocellulose membranes as cited above. For Western blot analyses, the primary antibodies anti-Rbt5 and anti-Enolase were used at 1/3,000 and 1/40,000 (v/v) ratios, respectively, and developed with BCIP-NBT, as cited above. Enolase was used as a negative control in this experiment.

Flow cytometry

Rbt5 binding affinities for hemoglobin and protoporphyrin were evaluated by flow cytometry. Yeast cells of *Paracoccidioides* [strains *Pb*01, *Pb*339 (*Pb*Wt), *Pb*rbt5-aRNA and *Pb*Wt+EV were cultivated as described above, washed with 1X PBS and blocked for 1 h at room temperature in 1X PBS, which was supplement with 1% bovine serum albumin (PBS-BSA). Fungal cells were then separated in two groups: the first group was initially treated with 20 μM protoporphyrin or 10 μM hemoglobin and further incubated with the anti-Rbt5 antibodies and an Alexa Fluor 488-labeled anti-rabbit IgG (10 μg/ml). The second group was sequentially incubated with primary and secondary antibodies as described above. The cells were then treated with 20 μM protoporphyrin or with 10 μM hemoglobin. All incubations were performed for 30 min at 37 °C, followed by washing with 1X PBS. Control cells were not exposed to hemoglobin or to protoporphyrin. Fluorescence levels of yeast cells were analyzed using a FACSCalibur (BD Biosciences) flow cytometer, and the data were processed using the FACS Express software.

Construction of *P. brasiliensis* Rbt5 antisense-RNA strain

The antisense-RNA (aRNA) strategy was used as described previously [47,65]. Briefly, DNA from wild-type *Pb*339 (*Pb*Wt) exponentially growing yeast cells was

obtained after cell rupture as described above. Platinum Taq DNA Polymerase High Fidelity (Invitrogen, USA) and the oligonucleotides as*rbt5*-S, 5' - CCGCTCGAGCGGTCTCGGAAACGACGGGTGC - 3' and as*rbt5*-AS, 5' - GGCGCGCCCGCAAGATTTCTCAACGCAAG - 3' were employed to amplify aRNA from *Pb*Wt *rbt5* DNA. Plasmid construction for aRNA gene repression and *A. tumefaciens*-mediated transformation (ATMT) of *Pb*Wt was performed as previously described [46,66]. The amplified *rbt5*-aRNA fragments were inserted into the pCR35 plasmid, which was flanked by the calcium-binding protein promoter region (P-*cbp-1*) from *H. capsulatum* and by the cat-B termination region (T-*cat-B*) of *Aspergillus fumigatus* [67]. The pUR5750 plasmid was used as a parental binary vector to harbor the aRNA cassette within the transfer DNA (T-DNA). The constructed binary vectors were introduced into *A. tumefaciens* LBA1100 ultracompetent cells by electroporation [68] and were isolated by kanamycin selection (100 mg/ml). The *A. tumefaciens* cells that were positive for pUR5750 transformation were used to perform the ATMT of *Paracoccidioides* yeast cells. The hygromycin (Hyg)-resistance gene, *hph*, from *E. coli* was used as a selection mark and was flanked by the glyceraldehyde-3-phosphate dehydrogenase promoter region (P-*gapdh*) and the trpC termination region (T-*trpC*) from *Aspergillus nidulans*. The selection of transformants (*Pbrbt5*-aRNA) was performed in BHI solid media with Hyg B (75 μg/ml Hyg) during 15 days of incubation at 36 °C. Randomly selected Hyg resistant transformants were tested for mitotic stability by subculturing the fungus three times in Hyg 75 μg/ml and three more times in Hyg 150 μg/ml. *Paracoccidioides* yeast cells were also transformed with the empty parental vector pUR5750 (*Pb*Wt+EV) as a control during the assays that were performed in this study. The investigation of *rbt5* gene expression was performed by qRT-PCR after consecutive subculturing.

Macrophage infection

Macrophages from the cell line J774 A.1 (BCRJ Cell Bank, Rio de Janeiro, accession number 0121), which were maintained in RPMI medium (RPMI 1640, Vitrocell, Brazil) that was supplemented with 10% (v/v) fetal bovine serum (FBS) at 37 °C in 5% CO_2, were used in this assay. In total, $1x10^6$ macrophages were seeded into each well of a 24-well tissue culture plate, and 100 U/ml of murine IFN-γ (PeproTech, Rocky Hill, New Jersey, USA) was added for 24 h at 37 °C in 5% CO_2 for macrophage activation as described previously [69]. Prior to co-cultivation, *Paracoccidioides* yeast

772 cells (*Pb*Wt, *Pbrbt5*-aRNA and *Pb*Wt+EV) were cultivated in BHI liquid medium for
773 72 h at 36 °C. For infection, $2.5x10^6$ *Paracoccidioides* yeast cells for each strain were
774 added to the macrophages independently. The cells were co-cultivated for 24 h at 37 °C
775 in 5% CO_2 to allow fungal internalization. Infected macrophages were first washed
776 three times with 1X PBS, and then macrophages were lysed with distilled water.
777 Dilutions of the lysates were plated in BHI medium, which was supplemented with 5%
778 (v/v) fetal bovine serum (FBS), at 36 °C. Colony forming units (CFUs) were counted
779 after growth for 10 days. CFUs were expressed as the mean value ± the standard error
780 of the mean (SEM) from triplicates, and statistical analyses were performed using
781 Student's t-test.

782 **BALB/c mice infection**

783 For the mouse infection experiment, *Pb*Wt, *Pbrbt5*-aRNA and *Pb*Wt+EV were
784 cultivated for 48 h in BHI medium, which was supplemented with 4% glucose. Thirty-
785 day-old male BALB/c mice (n = 4) were inoculated intraperitoneally with 10^7 yeast
786 cells of each strain independently, as previously described [42]. After 2 weeks of
787 infection, mouse spleens were removed and were homogenized using a grinder in 3 mL
788 of sterile 0.9% (w/v) NaCl. In total, 50 µl of the homogenized sample was plated on
789 BHI agar, which was supplemented with 4% (v/v) fetal calf serum and 4% (w/v)
790 glucose. The plates were prepared in triplicates for each organ of each animal and were
791 incubated at 36 °C. After 15 days, the CFUs for each organ that was infected with each
792 strain were determined by counting, and a mean for each condition was obtained. The
793 data were expressed as the mean value ± the SEM from quadruplicates, and statistical
794 analyses were performed using Student's t-test.

795

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799

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992 **Figure legends**

993 **Figure 1. Effect of different iron sources on the growth of *Paracoccidioides* yeast**
994 **cells.** *Pb*01 and *Pb*18 cell cultures were collected after 36 h of iron scarcity, washed and
995 ten-fold serial dilutions of cell suspensions (10^4 to 10 cells) were spotted on MMcM
996 plates, which were supplemented with 50 µM BPS, an iron chelator. As indicated,
997 different iron sources were added or not (no iron condition): 30 µM inorganic iron, 30
998 µM hemoglobin, 120 µM hemin, 30 µg/µl ferritin, 30 µM transferrin or 3 µM
999 lactoferrin.

1000 **Figure 2. *Paracoccidioides* can internalize protoporphyrin rings.** Iron deprived *Pb*01
1001 and *Pb*18 yeast cells were incubated in MMcM with (+) or without (-) 20 µM zinc
1002 protoporphyrin IX (Zn-PPIX) for 2 h. After this period, the cells were washed twice and
1003 observed by bright field microscopy (left panel of each strain) and by live fluorescence
1004 microscopy (right panel of each strain).

1005 **Figure 3. Hemolysis of sheep erythrocytes in the presence of *Paracoccidioides* yeast**
1006 **cells.** *Pb*01 and *Pb*18 10^7 yeast cell suspensions were incubated with 10^8 sheep
1007 erythrocytes for 2 h at 36 °C in 5% CO_2. As a negative or positive control, respectively,
1008 erythrocytes were incubated with phosphate buffered saline solution (PBS) or sterile
1009 water. The optical densities of the supernatants were determined with an ELISA plate
1010 reader at 405 nm. The experiment was performed in triplicate, and the average optical
1011 density of each condition was used to calculate the relative hemolysis of the
1012 experimental conditions or the negative control with respect to the positive control. The
1013 data are plotted as the mean $\pm$ standard deviation. *: statistically significant difference
1014 in comparison with PBS values according to Student's t-test.

1015 **Figure 4. Expression of genes that are putatively related to hemoglobin uptake.**
1016 *Pb*01 yeast cells were recovered from MMcM medium, which was supplemented or not
1017 (no iron addition condition) with different iron sources (10 µM and 100 µM inorganic
1018 iron and 10 µM hemoglobin) for 30, 60 and 120 min. After RNA extraction and cDNA
1019 synthesis, levels of *Pb*01 *rbt5*, *wap1* and *csa2* transcripts were quantified by qRT-PCR.
1020 The expression values were calculated using alpha tubulin as the endogenous control.
1021 The values that were plotted on the bar graph were normalized against the expression
1022 data that were obtained from the no iron addition condition (fold change). The data are

expressed as the mean ± SD of the triplicates. *statistically significant data as determined by Student's t-test ($p < 0.05$).

Figure 5. *Paracoccidioides* Rbt5 is a GPI-anchored protein localized in the yeast cell wall. A. Cell wall fraction of *Pb*01 yeast cells was obtained and analyzed by Western blot using polyclonal antibodies raised against the recombinant protein Rbt5. Proteins that were obtained from the cell wall (lane 1) were extracted by HF-pyridine digestion and analyzed (lane 2). Molecular weight markers are indicated at the right side of the panel. **B.** Immunoelectron microscopic detection of Rbt5 in *Pb*01 yeast cells by post embedding methods. (1) Negative control exposed to the rabbit preimmune serum. (2 and 3) Gold particles are observed at the fungus cell wall (arrow) and in the cytoplasm (double arrowheads). Bars: 1 µm (1 and 2) and 0.5 µm (3). v: vacuoles. m: mitochondria. w: cell wall.

Figure 6. *Paracoccidioides* Rbt5 binds heme-containing molecules. A. Recombinant proteins Rbt5 (lane 1) and Enolase (lane2), which was used as a negative control, were incubated with hemin-agarose resin. After 1 h, the resin was boiled with sample buffer, and the proteins were submitted to SDS-PAGE and Western blot analysis. A single 22 kDa immunoreactive species is present in lane 1, which corresponds to the Rbt5 recombinant protein. Enolase did not present an ability to bind hemin and, thus, was not purified with the resin. **B.** Upper and lower panels represent systems where protoporphyrin or hemoglobin Rbt5 recognition was assessed, respectively. The sequential steps of incubation in each system are indicated on the bottom of each panel. *Pb*01 denotes the background fluorescence of fungal cells alone; *Pb*01 + anti-Rbt5 represents systems where fungal cells were sequentially incubated with primary and secondary antibodies; *Pb*01 + protoporphyrin/hemoglobin + anti-Rbt5 is representative of systems that included the blocking of yeast cells with heme-containing molecules before exposure to antibodies; and *Pb*01 + anti-Rbt5 + protoporphyrin/hemoglobin represents systems that included the blocking of yeast cells with heme-containing molecules after exposure to antibodies.

Figure 7. *Paracoccidioides* Rbt5 knock down via an antisense-RNA (aRNA) strategy. A. Schematic representation of the T-DNA cassette that was used in this work to perform the *Agrobacterium tumefaciens*-mediated transformation (ATMT) of *Pb*339 (*Pb*Wt). *Pbrbt5*-aRNA was cloned in the pUR5750 binary vector under the control of

the *Histoplasma capsulatum cbp-1* gene promoter region (P-*cbp-1*) and the *Aspergillus fumigatus cat-B* gene termination region (T-*cat-B*). The selection marker that was used in this work was the *Escherichia coli* hygromycin-resistance gene *hph*. In the cassette, this gene is flanked by the glyceraldehyde-3-phosphate dehydrogenase promoter region (P-*gapdh*) and by the *trpC* termination region (T-*trpC*) from *Aspergillus nidulans*. **B.** After the selection of mitotic stable isolates, a qRT-PCR was performed to analyze the silencing level of the gene in isolates that were transformed with *Pbrbt5*-aRNA. As controls, *rbt5* transcript level from *Pb*Wt and *Pb*Wt transformed with the empty vector (*Pb*Wt+EV) were also quantified. Alpha tubulin was used as the endogenous control. The data are represented as the means ± SD from triplicate determinations. *: statistically significant data as determined by Student's t-test ($p < 0.05$) in comparison with the data that were obtained from *Pb*Wt+EV strain. **C.** Effect of *Pb*rbt5 deletion on the interaction of *Paracoccidioides* with heme-containing molecules. Hemoglobin prevents *Pb*Wt and *Pb*Wt+EV cells to be recognized by the anti-Rbt5 antibodies. However, *Pb*rbt5-aRNA cells are poorly recognized by the antibody that was raised against Rbt5, which is a process that was not affected by the previous or subsequent exposure of yeast cells to hemoglobin.

Figure 8. *Paracoccidioides* Rbt5 shows virulent and antigenic properties. A. To test the ability to infect macrophages, *Pb*Wt, *Pbrbt5*-aRNA or *Pb*Wt+EV strains were co-cultivated with macrophages for 24 h. After this period, infected macrophages were lysed, and lysates were plated on BHI medium to recover the fungi. The data are presented as a bar graph of the means ± SEM from triplicates. *: statistically significant data as determined by Student's t-test ($p < 0.05$) in comparison with the data that were obtained from the *Pb*Wt+EV strain. **B.** A murine model of infection was also used. Mice were infected intraperitoneally with *Pb*Wt, *Pbrbt5*-aRNA or *Pb*Wt+EV strains. After 2 weeks of infection, mice were sacrificed, the spleens were removed and samples of the homogenate were plated on BHI medium. After 15 days, the CFUs were counted to determine the fungal burden for each strain. The data are presented as a bar graph of the means ± SEM from quadruplicates. *: statistically significant data as determined by Student's t-test ($p < 0.05$) in comparison with the data that were obtained from the *Pb*Wt+EV strain. **C.** Reaction of the recombinant *Pb*01 Rbt5 with sera of five PCM patients (lanes 1-5) or with control sera (lanes 6-10). After reacting with the anti-human

IgG peroxidase coupled antibody, the reaction was developed using hydrogen peroxide and diaminobenzidine.

1089 Table 1. Some *Paracoccidioides Pb*01 proteins that were induced in the presence of hemoglobin as detected by nanoUPLC-MS^{E}.

Accession number[a]	Protein description	Score AVG	Peptides AVG	Fold change (Hb:Fe)	E.C. number	Subclassification	Filter[b]
METABOLISM							
Amino acid metabolismo							
PAAG_02163	acetyl-/propionyl-coenzyme A carboxylase alpha chain	249.65	16.33	***	6.4.1.3	Valine and isoleucine degradation	1
PAAG_07036	methylmalonate-semialdehyde dehydrogenase	279.14	14.83	1.60	1.2.1.27	Valine, leucine and isoleucine degradation	2
PAAG_00221	acetolactate synthase	260.71	10.20	1.55	2.2.1.6	Valine, leucine and isoleucine biosynthesis	2
PAAG_06416	conserved hypothetical protein (alanine racemase)	169.27	4.00	***	5.1.1.1	Alanine metabolism	1
PAAG_08065	aspartate-semialdehyde dehydrogenase	501.59	5.80	3.35	1.2.1.11	Amino acid biosynthesis	2
PAAG_03138	alanine-glyoxylate aminotransferase	381.01	7.33	1.40	2.6.1.44	Amino acid metabolism	2
PAAG_06217	acetylornithine aminotransferase	313.28	11.25	1.72	2.6.1.11	Arginine biosynthesis	1
PAAG_06506	aspartate aminotransferase	253.11	6.50	1.42	2.6.1.1	Aspartate and glutamate metabolism	1
PAAG_06835	cystathionine gamma-lyase	257.79	7.00	***	4.4.1.1	Cysteine biosynthesis	1

PAAG_07813	cysteine synthase	310.64	7.33	***	4.2.1.22	Cysteine biosynthesis	2
PAAG_05392	betaine aldehyde dehydrogenase	913.69	8.67	1.68	1.2.1.8	Glycine biosynthesis	2
PAAG_01568	glycine dehydrogenase	193.28	16.00	***	1.4.4.2	Glycine degradation	2
PAAG_05406	histidine biosynthesis trifunctional protein	196.65	14.00	***	3.5.4.19; 3.6.1.31; 1.1.1.23	Histidine biosynthesis	1
PAAG_00285	imidazole glycerol phosphate synthase hisHF	175.08	19.00	***	2.4.2.-; 4.1.3.-	Histidine biosynthesis	1
PAAG_09095	ATP phosphoribosyltransferase	1303.26	4.67	1.79	2.4.2.17	Histidine biosynthesis	2
PAAG_04099	methylcrotonoyl-CoA carboxylase subunit alpha	179.63	10.00	***	6.4.1.4	Leucine degradation	1
PAAG_06387	homoisocitrate dehydrogenase	431.93	8.00	1.84	1.1.1.87	Lysine biosynthesis	2
PAAG_02693	saccharopine dehydrogenase	241.77	12.67	1.43	1.5.1.10	Lysine metabolism	1
PAAG_07626	cobalamin-independent synthase	1296.09	25.33	2.92	2.1.1.14	Methionine biosynthesis	2
PAAG_06996	G-protein comlpex beta subunit CpcB	1162.11	11.33	2.32	N.A.	Regulation of amino acid metabolism	2
PAAG_03613	phosphoserine aminotransferase	265.03	8.67	4.35	2.6.1.52	Serine biosynthesis	2
PAAG_07760	threonine synthase	171.92	8.00	***	4.2.3.1	Threonine biosynthesis	1
PAAG_08668	anthranilate synthase component 2	242.62	11.50	***	4.1.3.27	Tryptophan biosynthesis	1

PAAG_05005	anthranilate synthase component 1	186.39	14.00	1.38	4.1.3.27	Tryptophan biosynthesis	2
PAAG_02644	kynurenine-oxoglutarate transaminase	179.07	6.00	***	2.6.1.7	Tryptophan degradation	2
PAAG_08164	homogentisate 1,2-dioxygenase	323.07	8.25	1.68	1.13.11.5	Tyrosine degradation	1
Nitrogen, sulfur and selenium metabolism							
PAAG_00468	4-aminobutyrate aminotransferase	1947.94	10.50	1.86	2.6.1.19	Nitrogen utilization	2
PAAG_03333	formamidase	14545.80	19.33	1.22	3.5.1.49	Nitrogen compound metabolic process	2
PAAG_05929	sulfate adenylyltransferase	303.58	9.20	2.39	2.7.7.4	Sulfur metabolism	2
PROTEIN FATE (folding, modification, destination)							
Protein/peptide degradation							
PAAG_02907	conserved hypothetical protein (ankyrin repeat protein)	288.17	6.00	***	N.A.	Cytoplasmic and nuclear protein degradation	1
PAAG_03512	carboxypeptidase Y	496.47	6.33	1.23	3.4.16.5	Lysosomal and vacuolar protein degradation	2
PAAG_01966	hypothetical protein (vacuolar protease A)	299.75	3.33	1.25	3.4.23.25	Lysosomal and vacuolar protein degradation	2
CELLULAR TRANSPORT, TRANSPORT FACILITIES AND TRANSPORT ROUTES							
Transported compounds							

PAAG_01051	conserved hypothetical protein (*Pb*01 Csa2)	151.83	6.00	***	N.A.	Hemoglobin receptor	1
INTERACTION WITH THE ENVIRONMENT							
Homeostasis							
PAAG_05851	cysteine desulfurase	160.27	7.00	***	2.8.1.7	Iron-sulfur cluster assembly	1
PAAG_05850	conserved hypothetical protein (cysteine desulfurase)	395.45	10.25	1.65	2.8.1.7	Iron-sulfur cluster assembly	1

[a]Information that was obtained from the *Paracoccidioides* Database (http://www.broadinstitute.org/annotation/genome/paracoccidioides_brasiliensis/MultiHome.html)

[b]filter 1 – proteins that were derived from PepFrag2; filter 2 – proteins that were derived from PepFrag1, as determined by PLGS and cited by Murad and Rech (2012).

***: proteins that were identified only in the presence of hemoglobin;

N.A.: not applicable.

1094 Table 2. Some *Paracoccidioides Pb*01 proteins that were repressed in the presence of hemoglobin as detected by nanoUPLC-MS^{E}.

Accession number	Protein description	Score AVG	Peptides AVG	Fold change (Hb:Fe)	E.C. number	Subclassification	Filter
METABOLISM							
Amino acid metabolism							
PAAG_01206	L-asparaginase	197.88	5.00	***	3.5.1.1	Asparagine degradation	1
PAAG_01365	choline dehydrogenase	206.25	13.00	***	1.1.99.1	Glycine biosynthesis	1
PAAG_02935	glycine cleavage system H protein	404.32	4.33	0.66	N.A.	Glycine degradation	2
PAAG_04102	isovaleryl-CoA dehydrogenase	330.74	7.00	***	1.3.8.4	Leucine degradation	2
PAAG_01974	mitochondrial methylglutaconyl-CoA hydratase	223.92	6.00	***	4.2.1.18	Leucine degradation	1
PAAG_03569	1,2-dihydroxy-3-keto-5-methylthiopentene dioxygenase	175.60	6.00	***	1.13.11.54	Methionine biosynthesis	1
PAAG_08166	4-hydroxyphenylpyruvate dioxygenase	203.44	3.00	***	1.13.11.27	Tyrosine and phenylalanine degradation	1
PAAG_00014	dihydroxy-acid dehydratase	312.52	10.83	0.73	4.2.1.9	Valine and isoleucine biosynthesis	2
PAAG_02554	3-hydroxyisobutyryl-CoA hydrolase	402.87	9.75	0.58	3.1.2.4	Valine degradation	2
PAAG_01194	2-oxoisovalerate dehydrogenase	219.86	6.00	***	1.2.4.4	Valine, leucine and isoleucine	2

	subunit beta					degradation	
PAAG_06096	phospho-2-dehydro-3-deoxyheptonate aldolase	1370.44	9.67	0.66	2.5.1.54	Phenyalanine, tyrosine and tryptophan biosynthesis	2
PAAG_07659	chorismate synthase	193.84	3.50	0.61	4.2.3.5	Phenyalanine, tyrosine and tryptophan biosynthesis	2
Nitrogen, sulfur and selenium metabolism							
PAAG_04525	glutamine synthetase	156.10	9.00	***	6.3.1.2	Nitrogen metabolism	1
PAAG_07689	NADP-specific glutamate dehydrogenase	2006.41	19.83	0.75	1.4.1.4	Nitrogen metabolism	2
Secondary metabolism							
PAAG_00799	uroporphyrinogen decarboxylase	362.04	8.67	***	4.1.1.37	Porphyrin biosynthesis	2
PAAG_06925	conserved hypothetical protein (glutamate-1-semialdehyde 2,1-aminomutase)	322.93	7.00	***	5.4.3.8	Porphyrin biosynthesis	2

[a]Information that was obtained from the *Paracoccidioides* Database (http://www.broadinstitute.org/annotation/genome/paracoccidioides_brasiliensis/MultiHome.html)

[b]filter 1 – proteins that were derived from PepFrag2; filter 2 – proteins that were derived from PepFrag1, as determined by PLGS and cited by Murad and Rech (2012).

***: proteins that were identified only in the presence of inorganic iron;

N.A.: not applicable.

Supporting Figure legends

Figure S1. Peptide error level that was obtained via the nanoUPLC-MS^E approach. The peptide and protein tables from PLGS were analyzed using the Spotfire software, which generated the ppm error graphics. These graphics indicate the number of peptides in a 15 ppm error range that were either obtained in the presence of 10 µM hemoglobin or obtained in the presence of 10 µM inorganic iron.

Figure S2. Peptide detection type that was used by the PLGS software. PLGS software uses an iterative search strategy for peptide identification as described previously [70]. During the first iteration (PepFrag1), only completely cleaved tryptic peptides are used for identification. The second pass of the database algorithm (PepFrag2) is designed to identify peptide modifications and nonspecific cleavage products to proteins that were positively identified in the first pass. VarMod: variable modifications. InSource: fragmentation that occurred on ionization source. MissedCleavage: missed cleavage performed by trypsin. NeutralLoss H_2O and NH_3 correspond to water and ammonia precursor losses. The Spotfire software was used to generate the graphics.

Figure S3. Expression and purification of the *Pb*01 recombinant Rbt5 and the generation of a rabbit polyclonal antibody. A. SDS-PAGE analysis of *Pb*01 recombinant Rbt5. *E. coli* cells harboring the pGEX-4T-3-Rbt5 plasmid were grown to an OD_{600} of 0.8 and harvested before (lane 1) or after (lane 2) incubation with IPTG. The cells were lysed by sonication, the recombinant protein was purified (lane 3) and the fusion protein (glutathione S-transferase, GST) was cleaved by thrombin digestion (lane 4). Electrophoresis was performed on 10% SDS–PAGE, and the proteins were stained by Coomassie blue R-250. **B and C**. Western blot analysis of the recombinant Rbt5. The proteins that were obtained were screened using the rabbit polyclonal antibody anti-rRbt5 **(B)** or the rabbit preimmune serum **(C)**. In B and C: *E. coli* C41 (DE3) that were transformed with the PGEX-4T-3-Rbt5 construct protein extract (lane 1); the affinity-isolated recombinant GST-Rbt5 (lane 2); the recombinant fusion protein cleaved with thrombin (lane 3). The reaction was developed using BCIP-NBT. Arrows indicate the deduced molecular mass of the proteins. Molecular markers are indicated at the left side of the panels.

Figure S4. *Pb*01 Rbt5 and *Candida* Rbt5 alignment. The amino acid sequences of the orthologs were aligned using the software ClustalX2. Asterisks: amino acid identity. Dots: conserved substitutions. In bold: signal peptide predicted by SignalP 4.1 Server. Grey box: CFEM domain that was predicted by the SMART online tool. In italic: cysteine residues inside the CFEM domain. Black border rectangle: omega-site that was predicted by the big-PI Fungal Predictor online tool.

Figure S5. *Paracoccidioides rbt5* knock down did not affect fungus growth under different iron availability conditions. *Pb*339 (*Pb*Wt), the *rbt5* knock down strain (*Pb*rbt5-aRNA) and the *Pb*339 strain that was transformed with the pUR5750 empty vector (*Pb*Wt+EV) were collected after 36 h of iron scarcity, washed, and ten-fold serial dilutions of cell suspensions (10^5 to 10^2 cells) were spotted on MMcM plates that were supplemented with 50 µM BPS, which is an iron chelator. As indicated, 30 µM inorganic iron or 30 µM hemoglobin were added or not (no iron condition).

Figure 1

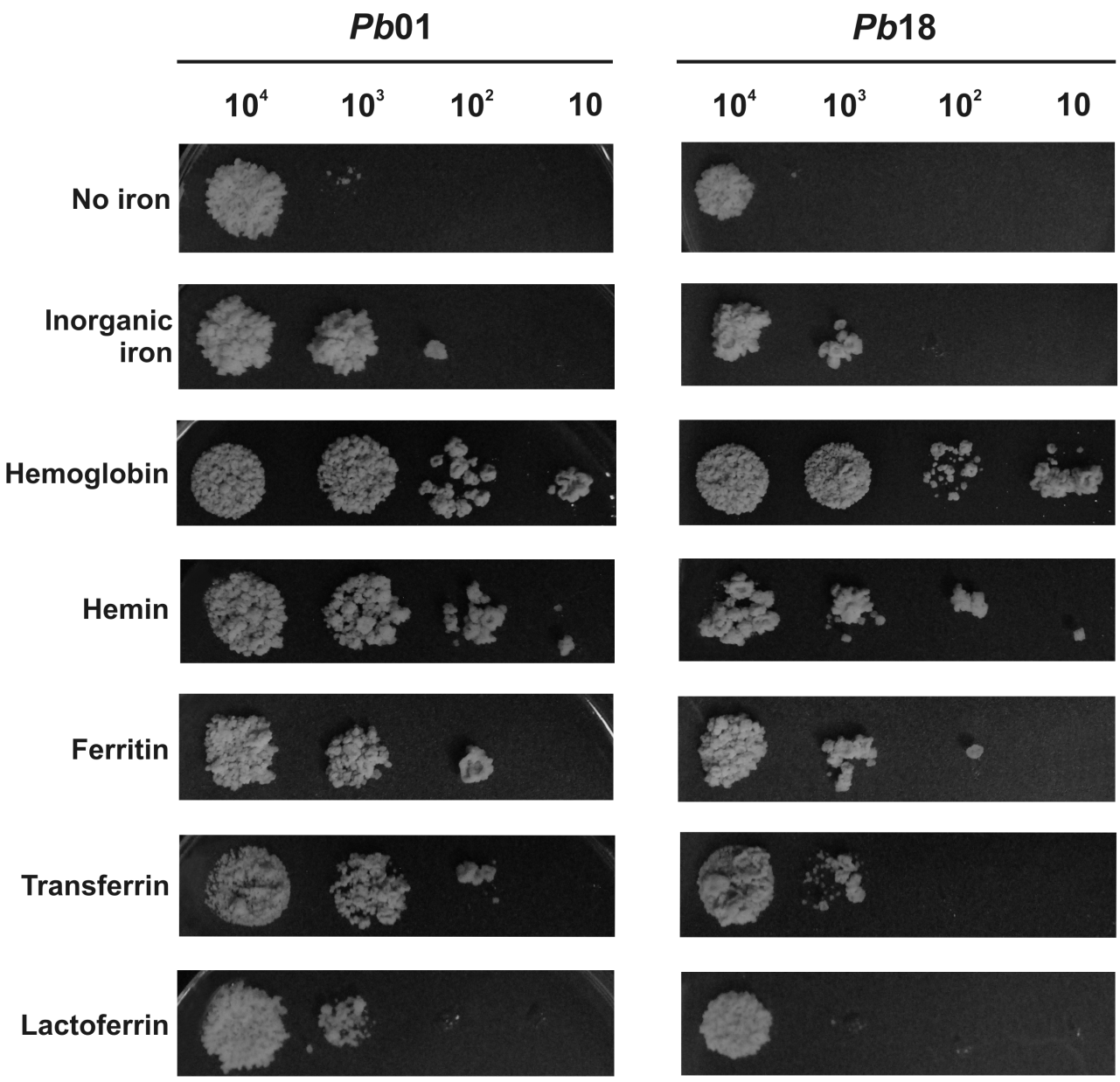

Pb01
Pb18
10^4
10^3
10^2
10
No iron
Inorganic iron
Hemoglobin
Hemin
Ferritin
Transferrin
Lactoferrin

Figure 2

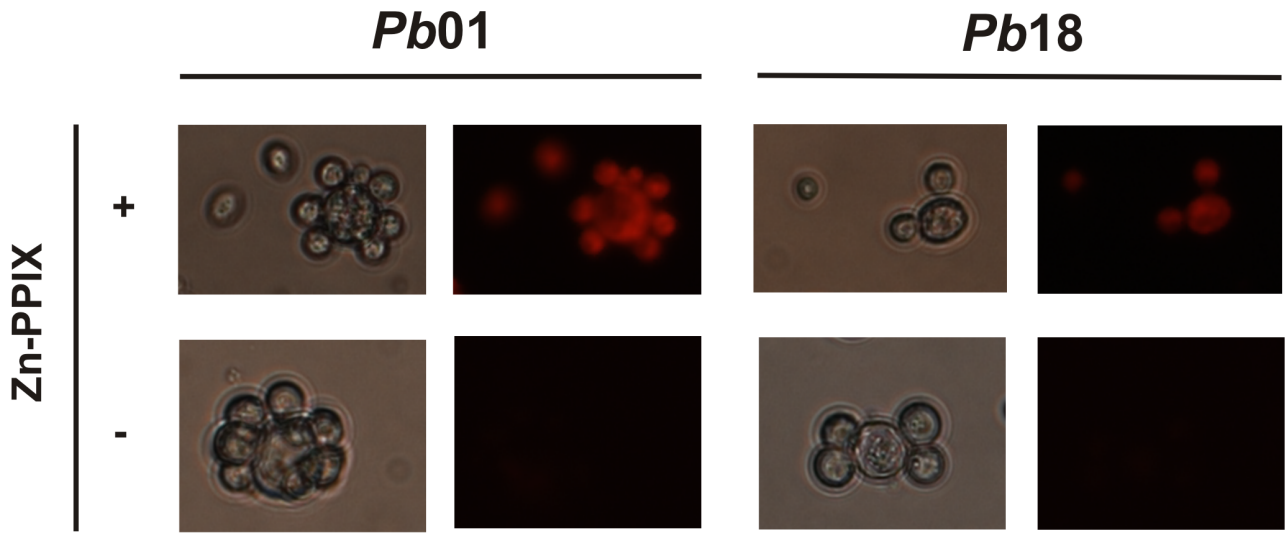


Figure 3

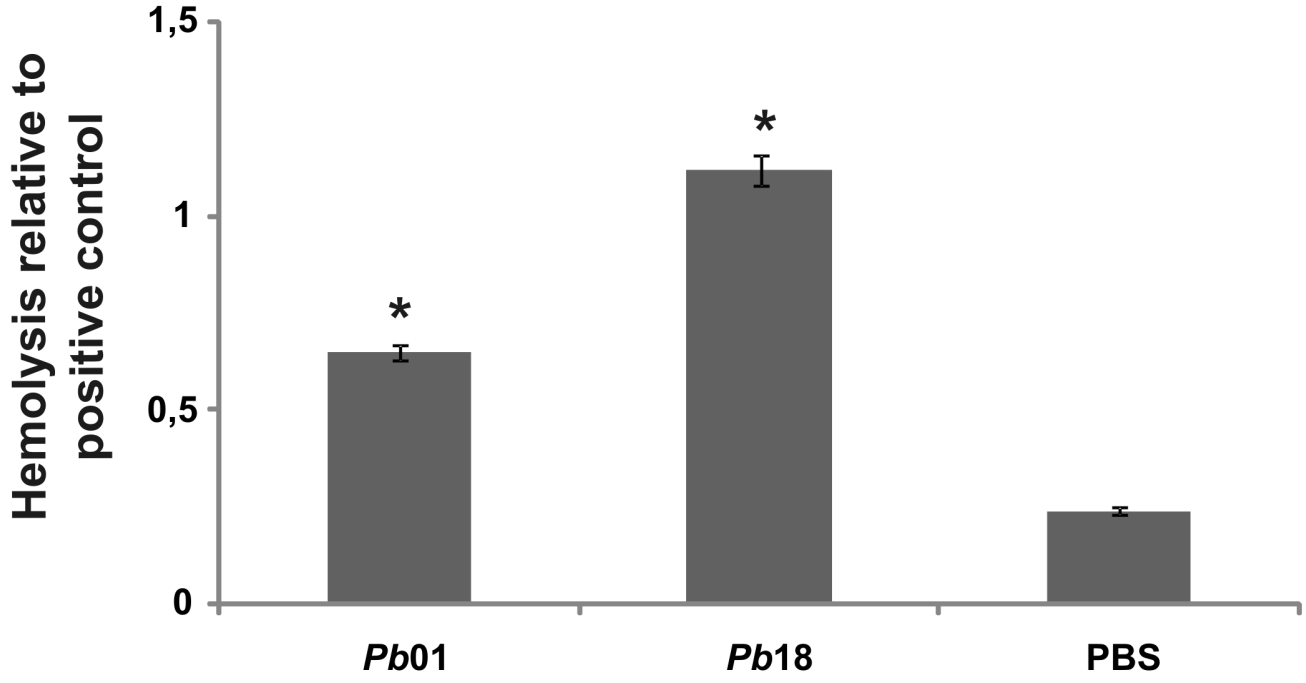

Hemolysis relative to positive control
1,5
1
0,5
0
*
*
*Pb*01
*Pb*18
PBS

Figure 4

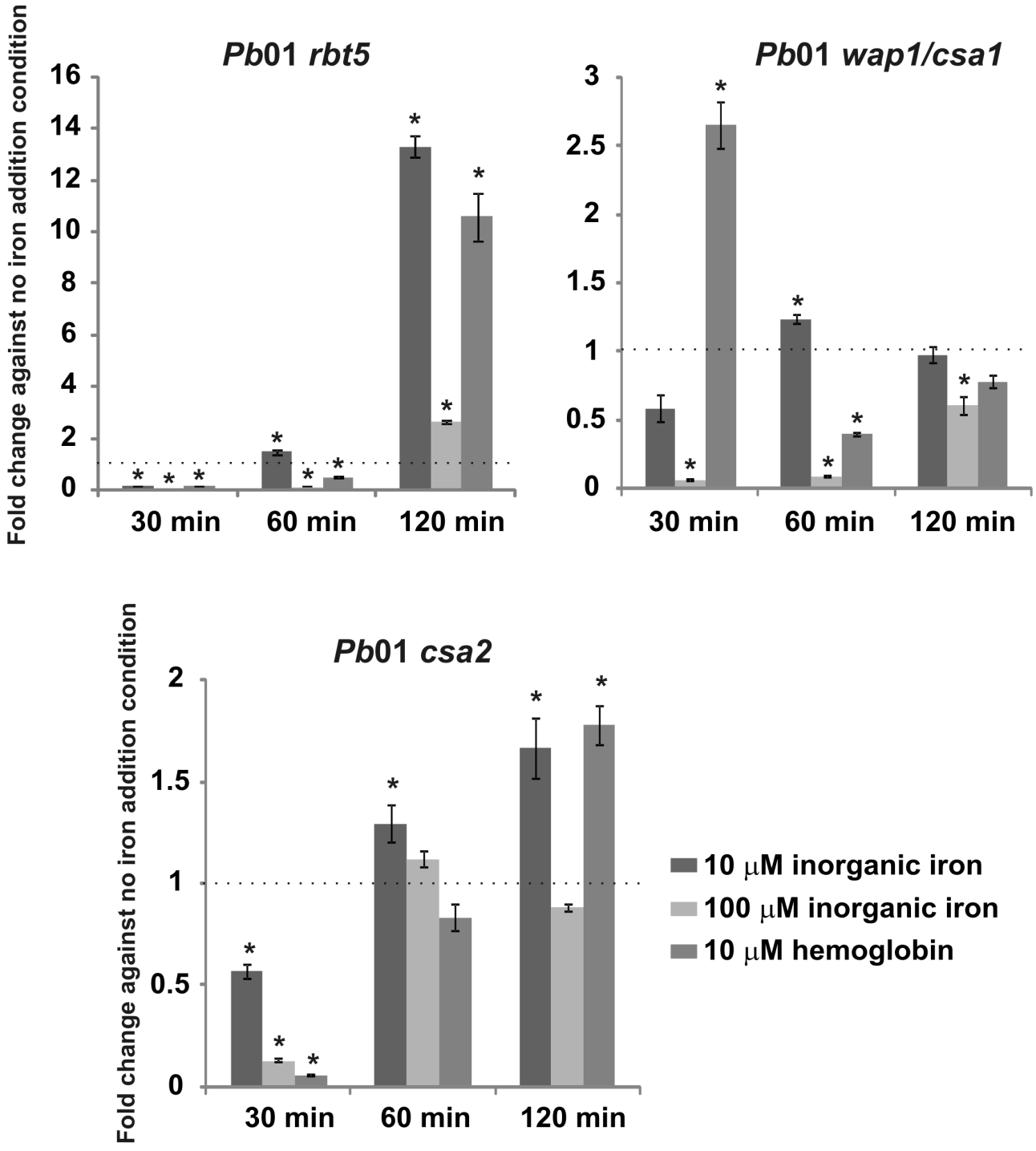

Pb01 rbt5
Pb01 wap1/csa1
Pb01 csa2
Fold change against no iron addition condition
30 min
60 min
120 min
10 μM inorganic iron
100 μM inorganic iron
10 μM hemoglobin

Figure 5

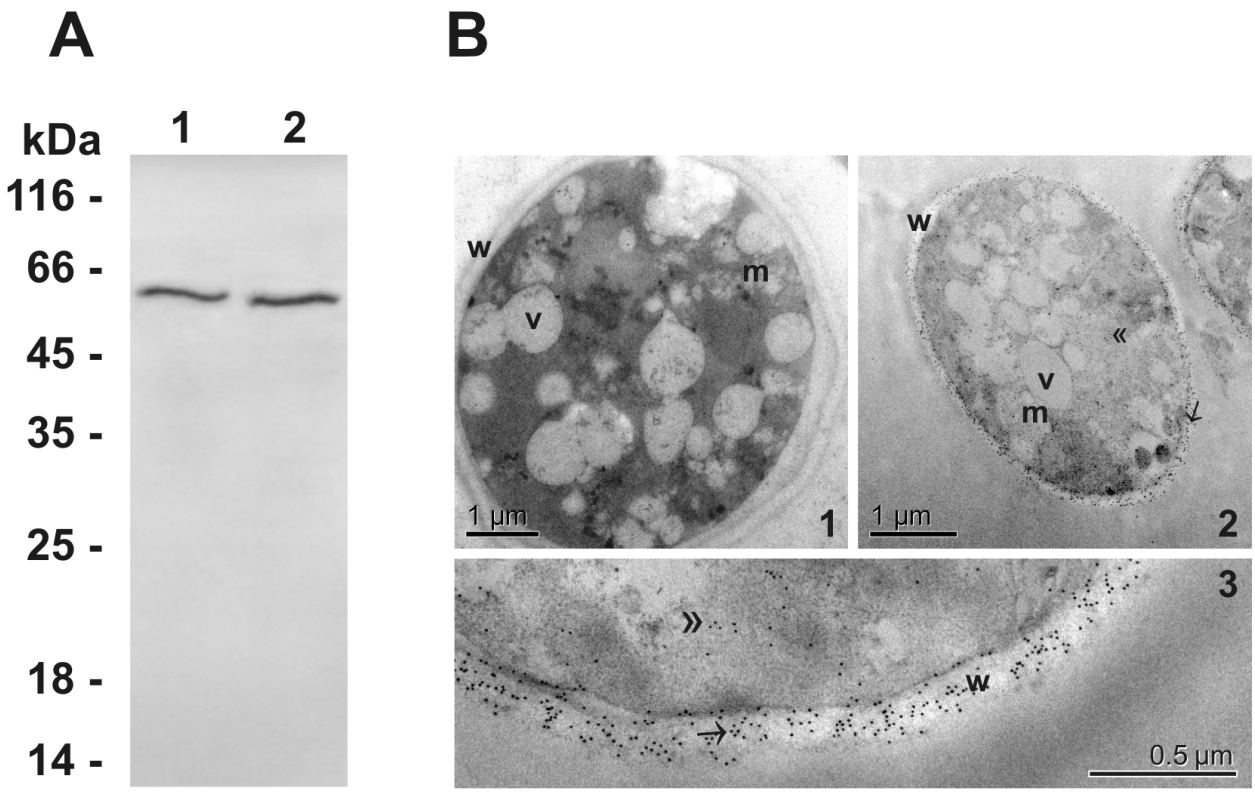


Figure 6

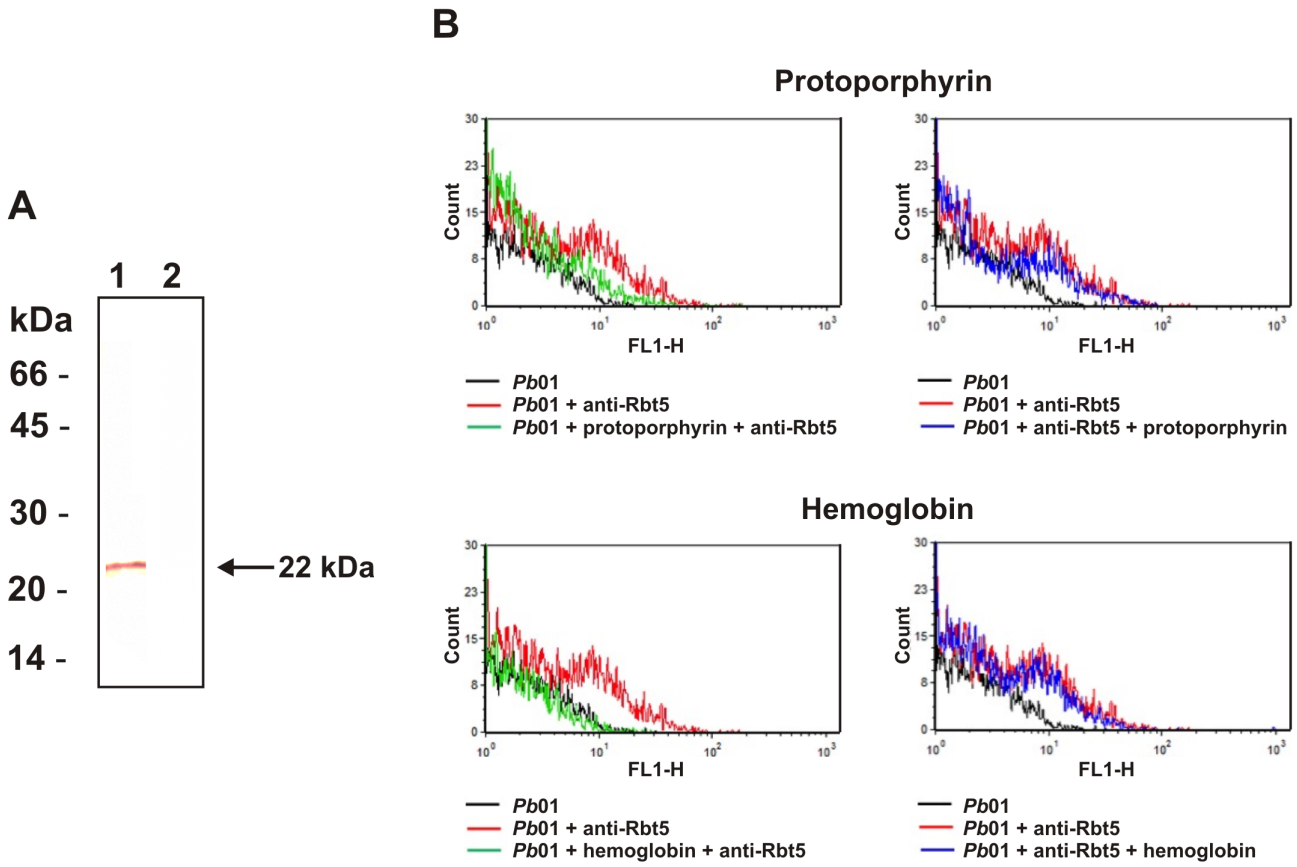

A
1 2
kDa
66 -
45 -
30 -
20 -
14 -
22 kDa
B
Protoporphyrin
Count
FL1-H
Pb01
Pb01 + anti-Rbt5
Pb01 + protoporphyrin + anti-Rbt5
Pb01 + anti-Rbt5 + protoporphyrin
Hemoglobin
Pb01 + hemoglobin + anti-Rbt5
Pb01 + anti-Rbt5 + hemoglobin

Figure 7

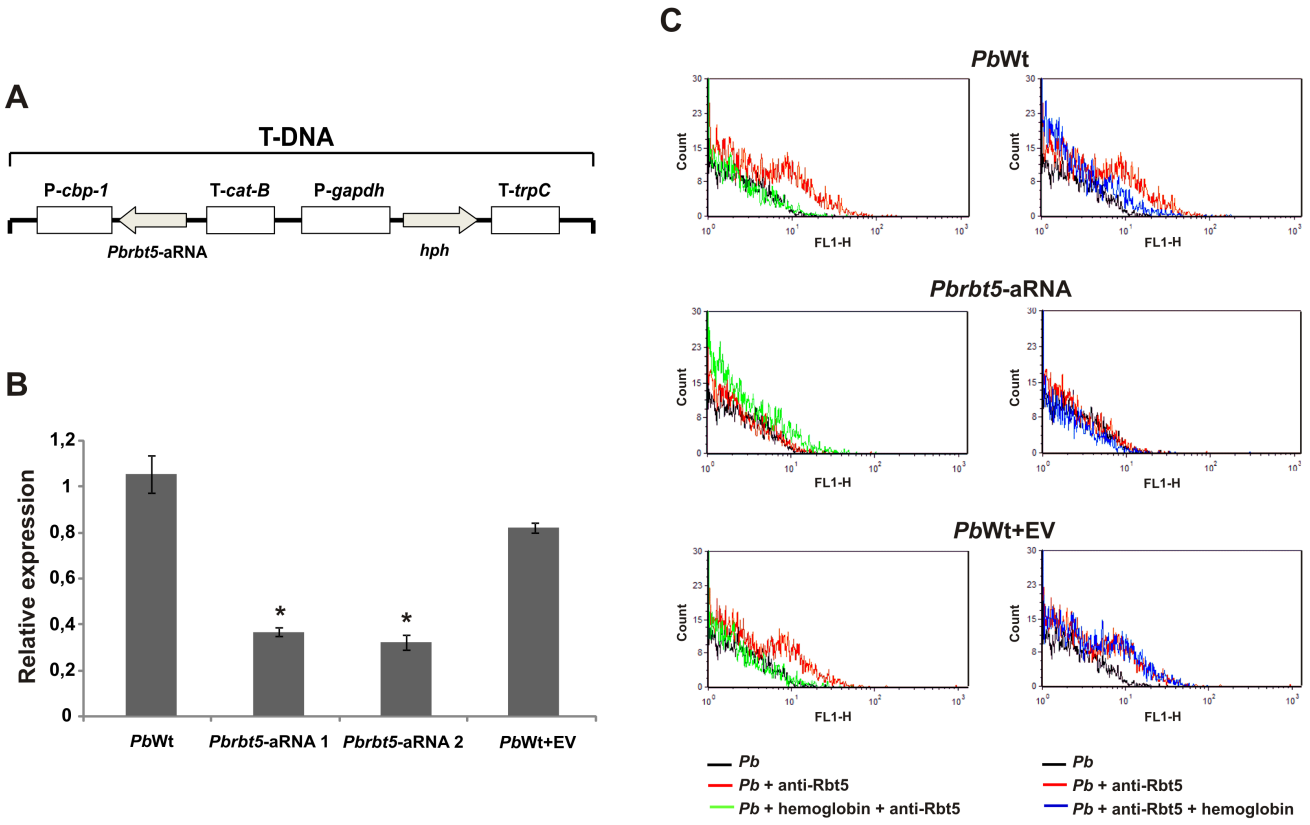
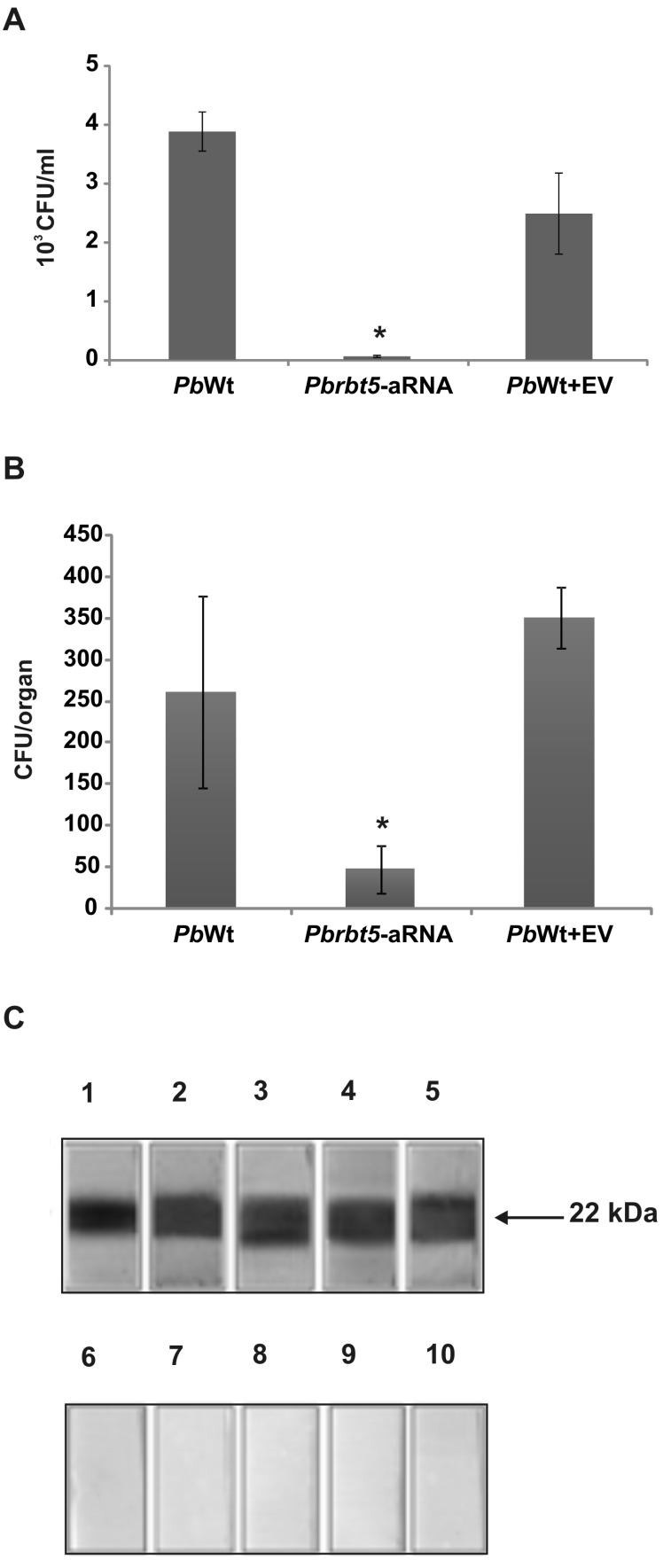
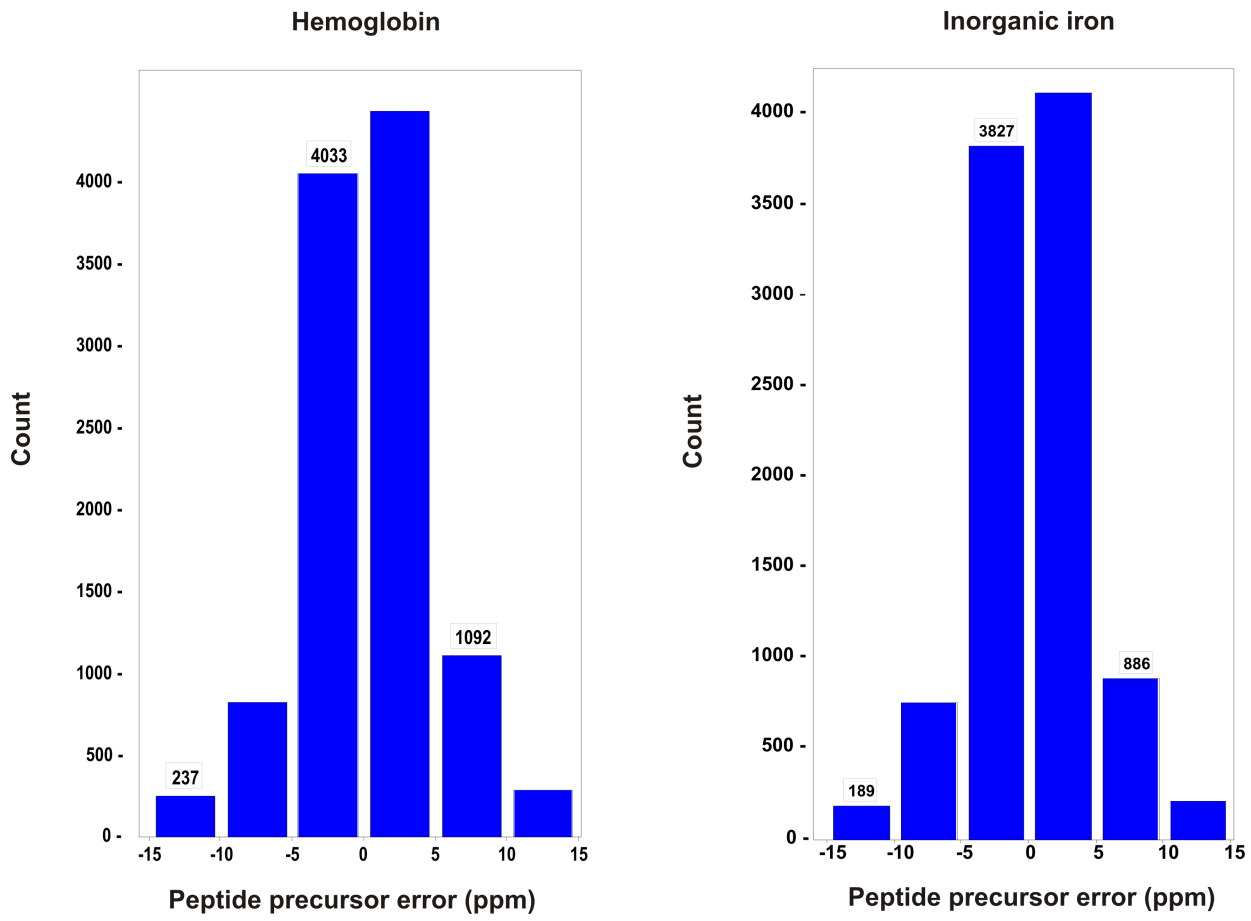

A
T-DNA
P-cbp-1
T-cat-B
P-gapdh
T-trpC
Pbrbt5-aRNA
hph
B
Relative expression
1,2
1
0,8
0,6
0,4
0,2
0
PbWt
Pbrbt5-aRNA 1
Pbrbt5-aRNA 2
PbWt+EV
C
PbWt
Pbrbt5-aRNA
PbWt+EV
Count
FL1-H
Pb
Pb + anti-Rbt5
Pb + hemoglobin + anti-Rbt5
Pb + anti-Rbt5 + hemoglobin

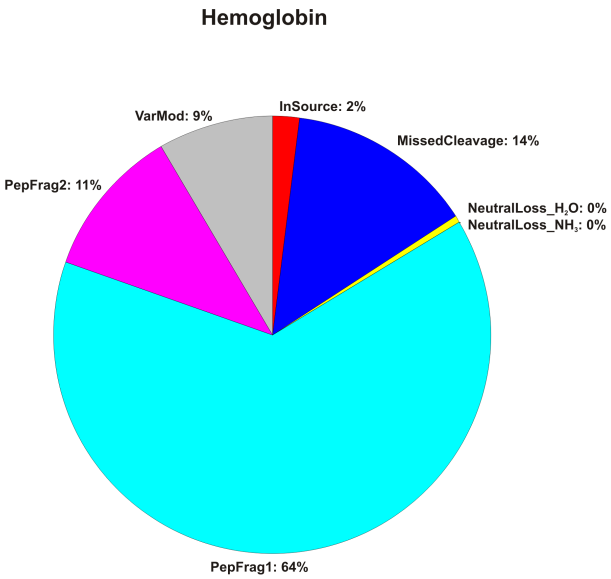
Figure 8

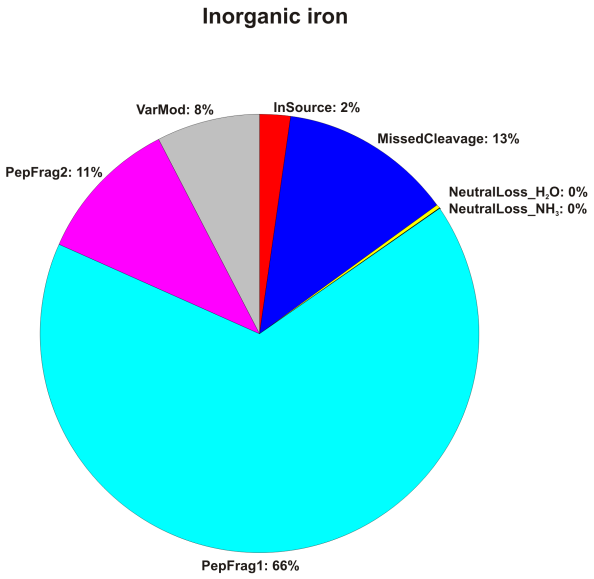

A
5
4
3
2
1
0
10³ CFU/ml
*
PbWt
Pbrbt5-aRNA
PbWt+EV
B
450
400
350
300
250
200
150
100
50
0
CFU/organ
*
PbWt
Pbrbt5-aRNA
PbWt+EV
C
1 2 3 4 5
22 kDa
6 7 8 9 10

Supporting Figure 1

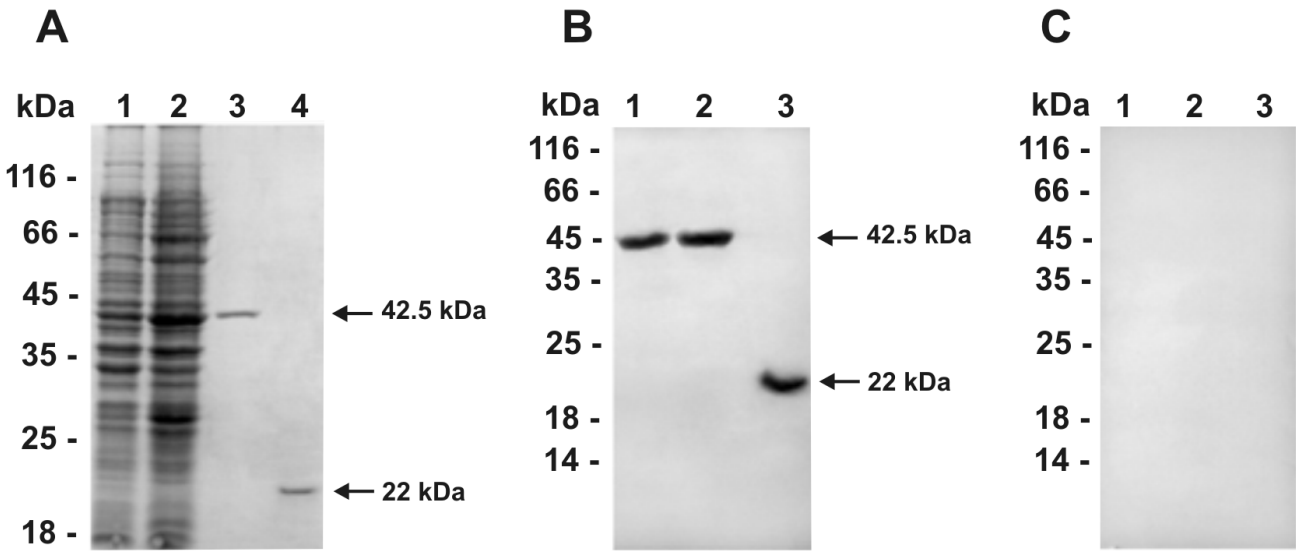


Supporting Figure 2

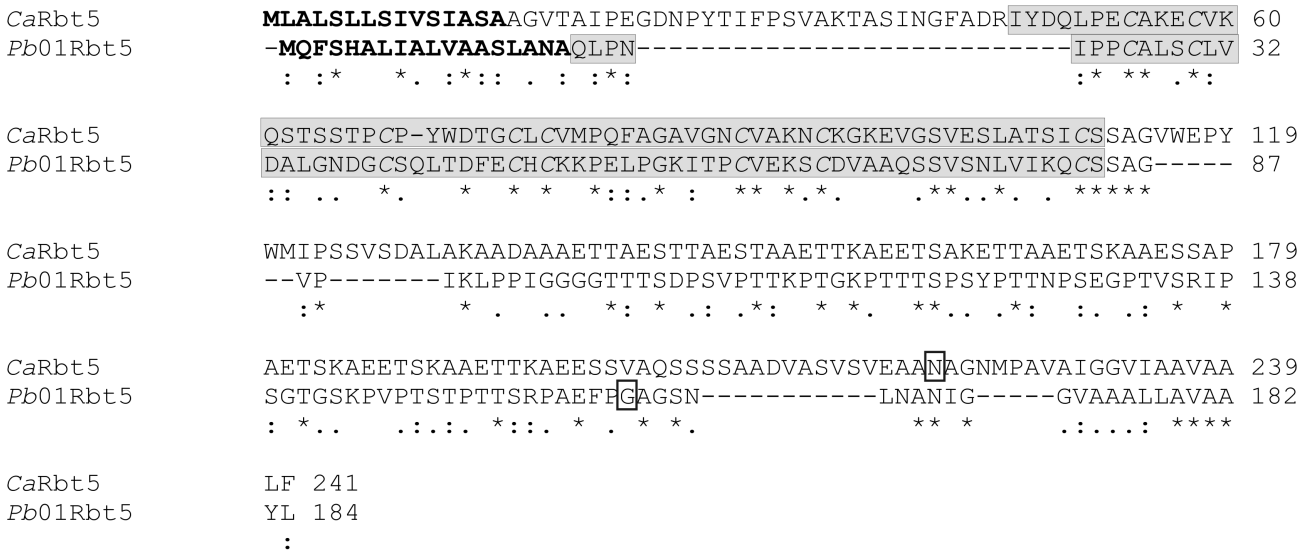




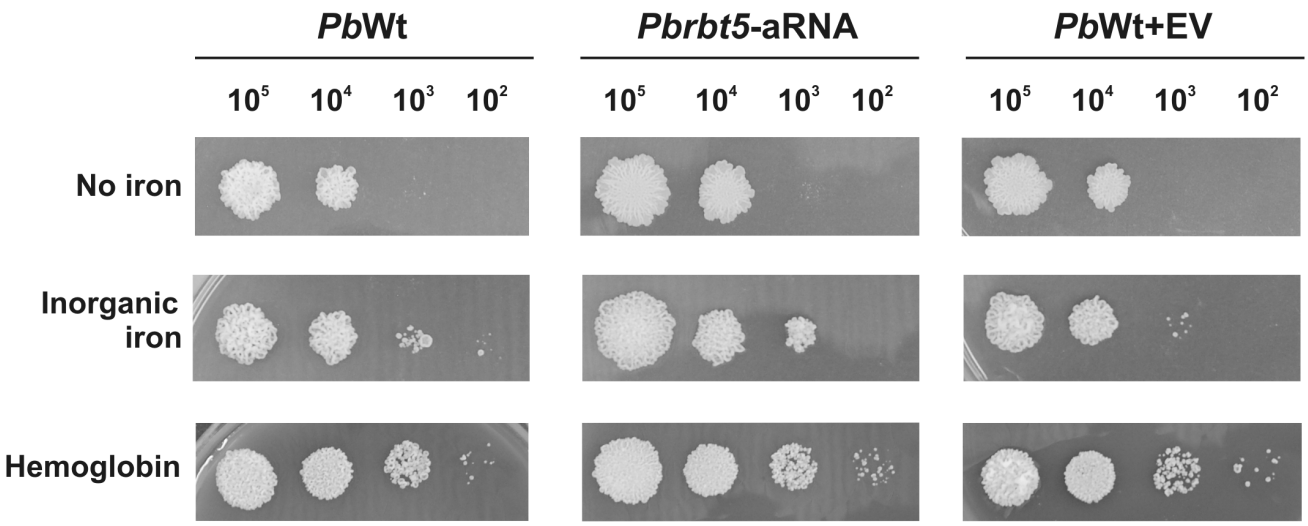
Supporting Figure 3



Supporting Figure 4



Supporting Figure 5



Supp. Table 1. Predicted members of the hemoglobin-receptor gene family in *Paracoccidioides* genus.

Orf[a]	Gene name	Amino acids number[a]	Signal peptide[b]	Transmembrane domain[c]	CFEM domain[c]	GPI-anchored (predicted)[d]
PAAG_05158	Rbt5	184	Yes	0	1	Yes
PAAG_02225	Csa1/ Wap1	453	No	0	1	Yes
PAAG_01051	Csa2	456	Yes	7	1	No
PADG_05363	Csa1/ Wap1	289	Yes	0	1	Yes
PABG_04599	Rbt51	184	Yes	0	1	Yes
PABG_01634	Csa2	475	No	5	1	No

[a]Information obtained from *Paracoccidioides* Database (http://www.broadinstitute.org/annotation/genome/paracoccidioides_brasiliensis/MultiHome.html).
Accession numbers: PAAG refers to *Pb*01, PADG refers to *Pb*18 and PABG refers to *Pb*03.
[b]*In silico* signal peptide prediction was performed with the online software SignalP 4.1 Server (http://www.cbs.dtu.dk/services/SignalP/).
[c]*In silico* protein analysis was performed with the online software SMART (http://smart.embl-heidelberg.de/smart/set_mode.cgi?NORMAL=1).
[d]GPI-anchor prediction was performed using the online software big-PI Fungal Predictor (http://mendel.imp.ac.at/gpi/fungi_server.html).

Supp. Table 2. *Paracoccidioides Pb*01 proteins induced in presence of hemoglobin.

Accession number[a]	Protein description	Score AVG	Peptides AVG	Fold change (Hb:Fe)	E.C. number	Subclassification	Filter[b]
METABOLISM							
Amino acid metabolism							
PAAG_02163	acetyl-/propionyl-coenzyme A carboxylase alpha chain	249.65	16.33	***	6.4.1.3	Valine and isoleucine degradation	1
PAAG_07036	methylmalonate-semialdehyde dehydrogenase	279.14	14.83	1.60	1.2.1.27	Valine, leucine and isoleucine degradation	2
PAAG_00221	acetolactate synthase	260.71	10.20	1.55	2.2.1.6	Valine, leucine and isoleucine biosynthesis	2
PAAG_06416	conserved hypothetical protein (alanine racemase)	169.27	4.00	***	5.1.1.1	Alanine metabolism	1
PAAG_08065	aspartate-semialdehyde dehydrogenase	501.59	5.80	3.35	1.2.1.11	Amino acid biosynthesis	2
PAAG_03138	alanine-glyoxylate aminotransferase	381.01	7.33	1.40	2.6.1.44	Amino acid metabolism	2
PAAG_06217	acetylornithine aminotransferase	313.28	11.25	1.72	2.6.1.11	Arginine biosynthesis	1
PAAG_06506	aspartate aminotransferase	253.11	6.50	1.42	2.6.1.1	Aspartate and glutamate metabolism	1
PAAG_06835	cystathionine gamma-lyase	257.79	7.00	***	4.4.1.1	Cysteine biosynthesis	1
PAAG_07813	cysteine synthase	310.64	7.33	***	4.2.1.22	Cysteine biosynthesis	2

PAAG_05392	betaine aldehyde dehydrogenase	913.69	8.67	1.68	1.2.1.8	Glicine biosynthesis	2
PAAG_01568	glycine dehydrogenase	193.28	16.00	***	1.4.4.2	Glycine degradation	2
PAAG_05406	histidine biosynthesis trifunctional protein	196.65	14.00	***	3.5.4.19; 3.6.1.31; 1.1.1.23	Histidine biosynthesis	1
PAAG_00285	imidazole glycerol phosphate synthase hisHF	175.08	19.00	***	2.4.2.-; 4.1.3.-	Histidine biosynthesis	1
PAAG_09095	ATP phosphoribosyltransferase	1303.26	4.67	1.79	2.4.2.17	Histidine biosynthesis	2
PAAG_04099	methylcrotonoyl-CoA carboxylase subunit alpha	179.63	10.00	***	6.4.1.4	Leucine degradation	1
PAAG_06387	homoisocitrate dehydrogenase	431.93	8.00	1.84	1.1.1.87	Lysine biosynthesis	2
PAAG_02693	saccharopine dehydrogenase	241.77	12.67	1.43	1.5.1.10	Lysine metabolism	1
PAAG_07626	Cobalamin-independent synthase	1296.09	25.33	2.92	2.1.1.14	Methionine biosynthesis	2
PAAG_06996	G-protein comlpex beta subunit CpcB	1162.11	11.33	2.32	N.A.	Regulation of amino acid metabolism	2
PAAG_03613	phosphoserine aminotransferase	265.03	8.67	4.35	2.6.1.52	Serine biosynthesis	2
PAAG_07760	threonine synthase	171.92	8.00	***	4.2.3.1	Threonine biosynthesis	1
PAAG_08668	anthranilate synthase component 2	242.62	11.50	***	4.1.3.27	Tryptophan biosynthesis	1
PAAG_05005	anthranilate synthase component 1	186.39	14.00	1.38	4.1.3.27	Tryptophan biosynthesis	2
PAAG_02644	kynurenine-oxoglutarate transaminase	179.07	6.00	***	2.6.1.7	Tryptophan degradation	2

PAAG_08164	homogentisate 1,2-dioxygenase	323.07	8.25	1.68	1.13.11.5	Tyrosine degradation	1
Nitrogen, sulfur and selenium metabolism							
PAAG_00468	4-aminobutyrate aminotransferase	1947.94	10.50	1.86	2.6.1.19	Nitrogen utilization	2
PAAG_03333	formamidase	14545.80	19.33	1.22	3.5.1.49	Nitrogen compound metabolic process	2
PAAG_05929	sulfate adenylyltransferase	303.58	9.20	2.39	2.7.7.4	Sulfur metabolism	2
Nucleotide/nucleoside/nucleobase metabolism							
PAAG_05018	allantoate amidohydrolase	261.39	8.00	***	3.5.3.9	Purine metabolism	2
PAAG_01751	conserved hypothetical protein (cytidine deaminase)	423.39	7.00	***	3.5.4.5	Pyrimidine metabolism	2
C-compound and carbohydrate metabolism							
PAAG_00050	pyruvate dehydrogenase protein X component	1118.49	11.50	1.93	N.A.	Acetyl-CoA biosynthesis	2
PAAG_03330	dihydrolipoyl dehydrogenase	2921.00	20.33	1.26	1.8.1.4	Acetyl-CoA biosynthesis	2
PAAG_01931	phosphoacetylglucosamine mutase	185.26	6.00	***	5.4.2.3	Chitin biosynthetic process	2
PAAG_06057	conserved hypothetical protein (aldose 1-epimerase)	280.05	4.00	***	5.1.3.3	Hexose metabolism	2
PAAG_03243	conserved hypothetical protein (aldose 1-epimerase)	627.33	6.83	1.28	5.1.3.3	Hexose metabolism	2
PAAG_01347	actin cytoskeleton protein (VIP1)	390.56	10.00	2.18	2.7.4.21; 2.7.4.24	Inositol phosphate biosynthesis	1

PAAG_04550	2-methylcitrate synthase	5518.20	20.17	4.53	2.3.3.5	Methylcitrate cycle	2
PAAG_04290	neutral alpha-glucosidase AB	218.88	13.33	1.35	3.2.1.20	Polysaccharide metabolism	1
PAAG_00435	(R)-benzylsuccinyl-CoA dehydrogenase	274.05	10.50	2.23	1.3.8.3	Toluene degradation	1
PAAG_06817	UTP-glucose-1-phosphate uridylyltransferase	445.48	14.67	***	2.7.7.9	UDP-glucose metabolic process	2
PAAG_02011	phosphoglucomutase	505.72	23.17	1.52	5.4.2.2	UDP-glucose metabolic process	2
Lipid, fatty acid and isoprenoid metabolism							
PAAG_07786	acetyl-CoA acetyltransferase	1409.07	7.83	1.73	2.3.1.9	Ergosterol biosynthesis	2
PAAG_03689	3-ketoacyl-CoA thiolase B	232.09	9.67	***	2.3.1.16	Fatty acid beta-oxidation	1
PAAG_05690	esterase D	227.18	7.00	***	3.1.1.1	Fatty acid biosynthesis	1
PAAG_07631	short chain dehydrogenase/reductase family protein	240.97	8.00	***	1.1.1.100	Fatty acid biosynthesis	2
PAAG_04811	2-hydroxyacyl-CoA lyase	236.87	10.50	***	4.1.-.-	Fatty acid metabolism	2
PAAG_06953	short chain dehydrogenase/reductase family	303.51	5.00	1.22	1.1.1.300	Glycolipid metabolism	2
PAAG_03960	isopentenyl-diphosphate Delta-isomerase	196.53	8.00	***	5.3.3.2	Isoprenoid metabolism	1
PAAG_06215	hydroxymethylglutaryl-CoA lyase	735.72	7.17	1.35	4.1.3.4	Lipid metabolic process	2
Metabolism of vitamins, cofactors and prosthetic groups							

PAAG_02352	molybdopterin binding domain-containing protein	167.28	6.00	***	N.A.	Molybdenum cofactor biosynthesis	1
PAAG_08856	nicotinate-nucleotide pyrophosphorylase	147.96	2.00	***	2.4.2.19	De novo NAD biosynthesis	1
Secondary metabolism							
PAAG_01244	beta-lactamase family protein	511.77	7.67	2.16	3.5.2.6	Beta-lactam degradation	2
PAAG_04478	dienelactone hydrolase family protein	512.91	7.20	1.77	3.1.1.45	Secondary metabolites biosynthesis	2
PAAG_04443	spermidine synthase	410.25	6.33	1.27	2.5.1.16	Amines metabolism	2
ENERGY							
Glycolysis and gluconeogenesis							
PAAG_08203	phosphoenolpyruvate carboxykinase	163.90	8.00	***	4.1.1.49	Gluconeogenesis	1
PAAG_06380	pyruvate kinase	1155.19	18.33	2.14	2.7.1.40	Glycolysis	2
PAAG_00771	enolase	41257.25	25.67	***	4.2.1.11	Glycolysis/ Gluconeogenesis	2
Pentose-phosphate pathway							
PAAG_01178	6-phosphogluconate dehydrogenase	1134.90	14.33	1.70	1.1.1.44	Oxidative branch	2
PAAG_04444	transketolase	1685.75	23.67	1.40	2.2.1.1	Non-oxidative branch	2
Tricarboxylic-acid cycle							
PAAG_07729	isocitrate dehydrogenase subunit 2	184.77	6.00	***	1.1.1.41	TCA cycle	1

PAAG_01725	succinate dehydrogenase flavoprotein subunit	223.67	8.50	***	1.3.5.1	TCA cycle	2
PAAG_02732	2-oxoglutarate dehydrogenase E1	276.45	18.25	1.62	1.2.4.2	TCA cycle	1
PAAG_08915	dihydrolipoamide succinyltransferase	749.88	13.33	1.27	2.3.1.61	TCA cycle	2
PAAG_00417	succinyl-CoA ligase subunit alpha	430.44	6.33	1.26	6.2.1.5	TCA cycle	2
Electron transport and membrane-associated energy conservation							
PAAG_02297	cytochrome b-c1 complex subunit Rieske	155.75	3.00	***	1.10.2.2	Electron transport chain	1
PAAG_04931	electron transfer flavoprotein subunit beta	389.96	7.00	***	N.A.	Electron transport chain	2
PAAG_08088	cytochrome b-c1 complex subunit 2	280.22	7.00	***	N.A.	Electron transport chain	2
PAAG_02382	quinone oxidoreductase	307.88	8.00	1.73	1.6.5.3	Electron transport chain	1
PAAG_06796	conserved hypothetical protein (cytochrome c oxidase subunit)	1238.58	6.50	1.20	1.9.3.1	Electron transport chain	2
Respiration							
PAAG_03309	suaprga1	1475.52	8.33	1.30	N.A.	Aerobic respiration	2
Fermentation							
PAAG_02050	pyruvate decarboxylase	2099.43	16.17	2.75	4.1.1.1	Alcoholic fermentation	2
PAAG_05249	aldehyde dehydrogenase	5638.29	23.50	2.16	1.2.1.3	Alcoholic fermentation	2
Energy conservation and regeneration							

PAAG_08082	plasma membrane ATPase	207.96	5.00	***	3.6.3.6	ATP biosynthesis	2
PAAG_05576	ATP synthase gamma chain	1480.68	7.80	2.03	3.6.3.14	ATP biosynthesis	2
PAAG_04820	ATPase alpha subunit	5155.57	21.50	1.92	3.6.3.14	ATP biosynthesis	2
PAAG_08037	ATP synthase subunit beta	8785.14	29.33	1.22	3.6.3.14	ATP biosynthesis	2
CELL RESCUE, DEFENSE AND VIRULENCE							
Stress response							
PAAG_02116	Hsp70	385.88	16.50	1.35	N.A.	Heat shock response	2
PAAG_08152	3',5'-bisphosphate nucleotidase	155.76	7.00	***	3.1.3.7	Hyperosmotic salinity response	1
PAAG_06947	gamma-glutamyltranspeptidase	204.85	12.00	***	2.3.2.2	Nitrogen starvation response	2
PAAG_00997	actin-interacting protein	178.28	12.00	***	N.A.	Osmotic stress response	1
PAAG_07020	thioredoxin reductase	340.22	7.00	***	1.8.1.9	Oxidative stress response	2
PAAG_05061	AhpC/TSA family protein	763.03	4.67	***	N.A.	Oxidative stress response	2
PAAG_09083	TCTP family protein	3094.32	6.83	1.72	N.A.	Oxidative stress response	2
PAAG_03931	glutathione S-transferase Gst3	1064.17	13.00	1.49	2.5.1.18	Oxidative stress response	2
CELL CYCLE AND DNA PROCESSING							
DNA processing							
PAAG_04389	mating-type switching protein swi10	224.32	6.00	***	N.A.	DNA repair	1

PAAG_07099	histone H3.3	1397.91	3.00	***	N.A.	DNA repair	2
PAAG_01596	chromatin remodeling complex subunit (Arp5)	176.87	13.00	***	N.A.	DNA repair	1
PAAG_00773	DNA damage checkpoint protein rad24	1991.61	9.83	3.90	N.A.	DNA repair	2
PAAG_06751	DNA damage checkpoint protein rad24	3506.98	13.00	2.51	N.A.	DNA repair	2
PAAG_00923	proliferating cell nuclear antigen	1264.29	9.00	1.60	N.A.	DNA synthesis and replication	2
PAAG_07098	histone H4.1	1662.13	5.00	***	N.A.	Nucleosome assembly	2
PAAG_00126	histone H4.2	1685.65	5.33	***	N.A.	Nucleosome assembly	2
Cell cycle							
PAAG_05518	cell division cycle protein	368.50	18.00	***	N.A.	Cell cycle	2
TRANSCRIPTION							
RNA synthesis							
PAAG_08471	histone H2A.Z	184.61	4.00	***	N.A.	Transcription regulation	2
PAAG_01710	polymerase II polypeptide D	245.71	4.00	***	2.7.7.6	Transcription	1
PROTEIN SYNTHESIS							
Ribosome biogenesis							
PAAG_01433	40S ribosomal protein S14	707.87	5.33	1.25	N.A.	Translation	2

PAAG_09043	40S ribosomal protein S2	1159.36	7.83	1.60	N.A.	Translation	2
PAAG_07847	40S ribosomal protein S26	1787.39	1.60	1.35	N.A.	Translation	2
PAAG_03828	40S ribosomal protein S9	1081.01	7.00	1.27	N.A.	Translation	2
PAAG_01052	60S ribosomal protein L10-B	671.85	5.80	1.43	N.A.	Translation	2
PAAG_06320	60S ribosomal protein L13	3696.57	8.50	1.26	N.A.	Translation	2
PAAG_00969	60S ribosomal protein L15	3234.57	6.33	1.28	N.A.	Translation	2
PAAG_01834	60S ribosomal protein L16	912.15	8.50	1.22	N.A.	Translation	2
PAAG_05379	60S ribosomal protein L17	2959.17	5.33	1.38	N.A.	Translation	2
PAAG_00430	60S ribosomal protein L2	2042.63	7.17	1.36	N.A.	Translation	2
PAAG_07385	60S ribosomal protein L23a	1911.74	4.67	1.20	N.A.	Translation	2
PAAG_00765	60S ribosomal protein L36	2645.63	6.33	1.28	N.A.	Translation	2
PAAG_07550	60S ribosomal protein L44	1230.65	3.00	***	N.A.	Translation	2
PAAG_06487	60S ribosomal protein L7-C	2051.46	9.50	1.25	N.A.	Translation	2
PAAG_04998	60S ribosomal protein L8-B	2603.35	12.83	1.28	N.A.	Translation	2
PAAG_00347	60S ribosomal protein L9-B	476.25	5.00	***	N.A.	Translation	2
PAAG_06743	ribosomal protein L23e	795.79	5.00	***	N.A.	Translation	2
PAAG_05051	conserved hypothetical protein (ribosomal protein S17)	188.74	6.00	***	N.A.	Translation	1

PAAG_01001	hypothetical protein (40S ribosomal protein S29)	217.83	3.67	1.28	N.A.	Translation	2
Translation							
PAAG_02921	elongation factor Tu	234.53	13.00	***	N.A.	Translation	2
PAAG_06084	eukaryotic translation initiation factor 3 subunit B	162.02	10.00	***	N.A.	Translation initiation	1
PAAG_03556	elongation factor 1-gamma 1	223.88	8.00	***	N.A.	Translation elongation	1
PAAG_00594	elongation factor 2	187.27	15.33	***	N.A.	Translation elongation	1
PAAG_01665	PKHD-type hydroxylase TPA1	156.35	7.00	***	1.14.11.-	Translation termination	1
PROTEIN FATE (folding, modification, destination)							
Protein folding and stabilization							
PAAG_06168	peptidyl-prolyl cis-trans isomerase cypE	805.88	5.17	1.36	5.2.1.8	Protein folding	2
PAAG_06255	mitochondrial co-chaperone GrpE	1865.43	8.20	1.35	N.A.	Protein folding	2
PAAG_05226	Hsp90 binding co-chaperone (Sba1)	3452.01	7.67	1.26	N.A.	Protein folding	2
PAAG_07409	conserved hypothetical protein (prefoldin subunit 6)	330.41	4.00	***	N.A.	Protein folding	2
Protein targeting, sorting and translocation							
PAAG_01854	hypothetical protein (nuclear transport factor 2)	621.37	1.50	***	N.A.	Protein import into nucleus	2

Protein modification							
PAAG_04195	ubiquitin-conjugating enzyme	250.71	1.00	***	N.A.	Ubiquitination	1
Protein/peptide degradation							
PAAG_02907	conserved hypothetical protein (ankyrin repeat protein)	288.17	6.00	***	N.A.	Cytoplasmic and nuclear protein degradation	1
PAAG_03512	carboxypeptidase Y	496.47	6.33	1.23	3.4.16.5	Lysossomal and vacuolar protein degradation	2
PAAG_01966	hypothetical protein (vacuolar protease A)	299.75	3.33	1.25	3.4.23.25	Lysossomal and vacuolar protein degradation	2
PROTEIN WITH BINDING FUNCTION OR COFACTOR REQUIREMENT							
Nucleic acid binding							
PAAG_05905	conserved hypothetical protein (dsDNA-binding protein PDCD5)	240.98	3.00	***	N.A.	DNA binding	1
PAAG_04913	conserved hypothetical protein (RNP domain-containing protein)	2396.12	9.83	1.43	N.A.	DNA binding	2
Structural protein binding							
PAAG_00004	actin binding protein	570.45	12.83	2.01	N.A.	Actin filament binding	2
PAAG_01764	actin binding protein	288.22	9.00	1.58	N.A.	Actin filament binding	1
Metal binding							
PAAG_03314	RING finger domain-containing	157.32	4.00	***	N.A.	Zinc binding	1

	protein						
PAAG_00976	LiPid Depleted family member	195.69	5.00	***	N.A.	Iron binding	1
Nucleotide/nucleoside/nucleobase binding							
PAAG_05623	conserved hypothetical protein (protein kinase)	208.36	9.00	***	N.A.	ATP binding	2
CELLULAR TRANSPORT, TRANSPORT FACILITIES AND TRANSPORT ROUTES							
Transported compounds							
PAAG_04276	phosphatidylinositol transporter	1310.76	10.83	1.65	N.A.	Golgi protein transport	2
PAAG_01051	conserved hypothetical protein (*Pb*01 Csa2)	151.83	6.00	***	N.A.	Hemoglobin receptor	1
INTERACTION WITH THE ENVIRONMENT							
Homeostasis							
PAAG_05851	cysteine desulfurase	160.27	7.00	***	2.8.1.7	Iron-sulfur cluster assembly	1
PAAG_05850	conserved hypothetical protein (cysteine desulfurase)	395.45	10.25	1.65	2.8.1.7	Iron-sulfur cluster assembly	1
BIOGENESIS OF CELLULAR COMPONENTS							
Cell wall							
PAAG_07670	cell wall protein ECM33 precursor	347.01	6.67	***	N.A.	Cell wall organization	2
PAAG_05068	1,3-beta-glucanosyltransferase gel4	159.84	10.00	***	2.4.1.-	Cell wall organization	1
PAAG_05763	conserved hypothetical protein (acid	456.10	7.50	1.92	3.1.3.2	Anchored to membrane	2

	phosphatase)						
UNCLASSIFIED PROTEINS							
PAAG_08235	predicted protein	393.54	5.00	***	N.A.	N.A.	1
PAAG_07397	conserved hypothetical protein	185.29	5.00	***	N.A.	N.A.	1
PAAG_07322	predicted protein	268.59	2.00	***	N.A.	N.A.	1
PAAG_06963	hypothetical protein	244.60	1.00	***	N.A.	N.A.	1
PAAG_05977	predicted protein	216.15	4.00	***	N.A.	N.A.	2
PAAG_05578	predicted protein	451.29	6.00	***	N.A.	N.A.	2
PAAG_02285	predicted protein	195.50	4.00	***	N.A.	N.A.	1
PAAG_01245	predicted protein	306.96	2.00	***	N.A.	N.A.	1
PAAG_00798	predicted protein	257.69	6.00	***	N.A.	N.A.	1
PAAG_00324	conserved hypothetical protein	241.69	7.00	***	N.A.	N.A.	1
PAAG_00250	conserved hypothetical protein	357.98	1.00	***	N.A.	N.A.	1
PAAG_00090	conserved hypothetical protein	335.29	6.00	***	N.A.	N.A.	2
PAAG_01045	conserved hypothetical protein	375.12	3.67	4.01	N.A.	N.A.	2
PAAG_03066	conserved hypothetical protein (putative cyclase)	982.37	7.83	1.88	N.A.	N.A.	2
PAAG_03701	BAR domain-containing protein	628.35	9.00	1.60	N.A.	N.A.	2

PAAG_07875	conserved hypothetical protein	11074.60	8.17	1.20	N.A.	N.A.	2

[a]Information obtained from *Paracoccidioides* Database (http://www.broadinstitute.org/annotation/genome/paracoccidioides_brasiliensis/MultiHome.html)
[b]filter 1 – proteins derived from PepFrag2; filter 2 – proteins derived from PepFrag1, as determined by PLGS and cited by Murad and Rech (2012).
***: proteins identified just in presence of hemoglobin;
N.A.: not applicable.

Supp. Table 3. *Paracoccidioides Pb*01 proteins repressed in presence of hemoglobin.

Accession number	Protein description	Score AVG	Peptides AVG	Fold change (Hb:Fe)	E.C. number	Subclassification	Filter
METABOLISM							
Amino acid metabolism							
PAAG_01206	L-asparaginase	197.88	5.00	***	3.5.1.1	Asparagine degradation	1
PAAG_01365	choline dehydrogenase	206.25	13.00	***	1.1.99.1	Glycine biosynthesis	1
PAAG_02935	glycine cleavage system H protein	404.32	4.33	0.66	N.A.	Glycine degradation	2
PAAG_04102	isovaleryl-CoA dehydrogenase	330.74	7.00	***	1.3.8.4	Leucine degradation	2
PAAG_01974	mitochondrial methylglutaconyl-CoA hydratase	223.92	6.00	***	4.2.1.18	Leucine degradation	1
PAAG_03569	1,2-dihydroxy-3-keto-5-methylthiopentene dioxygenase	175.60	6.00	***	1.13.11.54	Methionine biosynthesis	1
PAAG_08166	4-hydroxyphenylpyruvate dioxygenase	203.44	3.00	***	1.13.11.27	Tyrosine and phenylalanine degradation	1
PAAG_00014	dihydroxy-acid dehydratase	312.52	10.83	0.73	4.2.1.9	Valine and isoleucine biosynthesis	2
PAAG_02554	3-hydroxyisobutyryl-CoA hydrolase	402.87	9.75	0.58	3.1.2.4	Valine degradation	2
PAAG_01194	2-oxoisovalerate dehydrogenase subunit beta	219.86	6.00	***	1.2.4.4	Valine, leucine and isoleucine degradation	2

PAAG_06096	phospho-2-dehydro-3-deoxyheptonate aldolase	1370.44	9.67	0.66	2.5.1.54	Phenyalanine, tyrosine and tryptophan biosynthesis	2
PAAG_07659	chorismate synthase	193.84	3.50	0.61	4.2.3.5	Phenyalanine, tyrosine and tryptophan biosynthesis	2
Nitrogen, sulfur and selenium metabolism							
PAAG_04525	glutamine synthetase	156.10	9.00	***	6.3.1.2	Nitrogen metabolism	1
PAAG_07689	NADP-specific glutamate dehydrogenase	2006.41	19.83	0.75	1.4.1.4	Nitrogen metabolism	2
Nucleotide/nucleoside/nucleobase metabolism							
PAAG_04297	thymidylate kinase	359.06	5.00	***	2.7.4.9	Pyrimidine biosynthesis	2
PAAG_02336	conserved hypothetical protein (NUDIX hydrolase)	238.53	8.00	***	3.6.1.-	Nucleoside diphosphate metabolism	1
C-compound and carbohydrate metabolism							
PAAG_01534	pyruvate dehydrogenase E1 component subunit beta	289.61	5.83	0.74	1.2.4.1	Acetyl-CoA biosynthesis	2
PAAG_05761	4-carboxymuconolactone decarboxylase family protein	231.12	2.00	***	4.1.1.44	Aromatic hydrocarbons catabolism	1
PAAG_05572	conserved hypothetical protein (endo-1,3(4)-beta-glucanase)	504.06	4.67	***	3.2.1.39	Carbohydrate metabolism	2
PAAG_06473	mannitol-1-phosphate 5-dehydrogenase	3302.60	16.33	0.78	1.1.1.17	Fructose and mannose metabolism	2
PAAG_03776	inositol-3-phosphate synthase	1569.78	15.33	0.79	5.5.1.4	Inostiol biosynthesis	2

PAAG_04181	sorbitol utilization protein SOU2	662.02	4.83	0.54	N.A.	Sorbitol metabolism	2
Lipid, fatty acid and isoprenoid metabolism							
PAAG_06329	3-hydroxybutyryl-CoA dehydrogenase	427.12	8.00	***	1.1.1.157	Fatty acid metabolism	2
PAAG_05837	palmitoyl-protein thioesterase	280.07	15.00	***	3.1.2.22	Fatty acid elongation	2
PAAG_02664	3-ketoacyl-CoA thiolase	2491.03	11.17	0.80	2.3.1.16	Fatty acid metabolism	2
PAAG_06309	enoyl-CoA hydratase	2395.39	11.50	0.70	4.2.1.17	Fatty acid metabolism	2
PAAG_01928	peroxisomal dehydratase	370.76	8.75	0.37	N.A.	Fatty acid metabolism	2
Metabolism of vitamins, cofactors and prosthetic groups							
PAAG_01324	folic acid synthesis protein	351.68	10.80	0.77	4.1.2.25; 2.7.6.3; 2.5.1.15	Folic acid biosynthesis	2
PAAG_07321	pyridoxine biosynthesis protein PDX1	2963.57	12.50	0.75	4.-.-.-	Pyridoxine biosynthesis	2
Secondary metabolism							
PAAG_08820	conserved hypothetical protein (tyrosinase)	205.13	8.75	0.79	1.14.18.1	Melanin biosynthesis	1
PAAG_04888	4-coumarate-CoA ligase	190.87	9.00	***	6.2.1.12	Phenylpropanoids biosynthesis	1
PAAG_00799	uroporphyrinogen decarboxylase	362.04	8.67	***	4.1.1.37	Porphyrin biosynthesis	2
PAAG_06925	conserved hypothetical protein (glutamate-1-semialdehyde 2,1-	322.93	7.00	***	5.4.3.8	Porphyrin biosynthesis	2

	aminomutase)						
ENERGY							
Glycolysis and gluconeogenesis							
PAAG_08468	glyceraldehyde-3-phosphate dehydrogenase	22116.30	24.67	0.58	1.2.1.12	Glycolysis/ Gluconeogenesis	2
Tricarboxylic-acid cycle							
PAAG_00053	malate dehydrogenase	29621.08	24.50	0.77	1.1.1.37	TCA cycle	2
Electron transport and membrane-associated energy conservation							
PAAG_06268	cytochrome c	585.65	3.80	0.70	N.A.	Electron transport chain	2
PAAG_02699	hypothetical protein (ubiquinol-cytochrome c reductase subunit 6)	1636.99	2.67	0.79	N.A.	Electron transport chain	2
PAAG_07921	conserved hypothetical protein (putative cytochrome c oxidase subunit vib protein)	1384.39	2.60	0.63	N.A.	Electron transport chain	2
Fermentation							
PAAG_08911	alcohol dehydrogenase	315.73	9.00	***	1.1.1.1	Alcoholic fermentation	2
PAAG_04541	alcohol dehydrogenase	3967.62	12.67	0.80	1.1.1.1	Alcoholic fermentation	2
PAAG_00403	alcohol dehydrogenase	14799.24	21.67	0.59	1.1.1.1	Alcoholic fermentation	2
Energy conservation and regeneration							
PAAG_05605	ATP synthase delta chain	1667.22	5.33	0.80	3.6.3.14	ATP biosynthesis	2

PAAG_02019	conserved hypothetical protein (mitochondrial F1F0 ATP synthase subunit Atp14)	1075.80	3.00	0.54	3.6.3.14	ATP biosynthesis	2
CELL RESCUE, DEFENSE AND VIRULENCE							
Stress response							
PAAG_02725	superoxide dismutase	508.63	5.00	***	1.15.1.1	Oxidative stress response	2
PAAG_03292	cytochrome c peroxidase	371.91	7.83	0.70	1.11.1.5	Oxidative stress response	2
PAAG_01454	catalase	781.72	5.20	0.37	1.11.1.6	Oxidative stress response	2
PAAG_00293	quinone oxidoreductase	232.23	4.00	***	1.6.5.5	Oxidative stress response	1
CELL CYCLE AND DNA PROCESSING							
DNA processing							
PAAG_08918	late histone H2B.L4	3411.59	5.00	0.67	N.A.	Nucleosome assembly	2
PAAG_08917	histone H2a	1669.12	4.83	0.59	N.A.	Nucleosome assembly	2
PAAG_00427	conserved hypothetical protein (DNA repair and transcription factor Ada)	152.88	5.00	***	N.A.	DNA repair	1
Cell cycle							
PAAG_02186	nuclear segregation protein Bfr1	398.41	11.00	0.73	N.A.	Mitosis regulation	2
PAAG_03054	conserved hypothetical protein (G2/M phase checkpoint control protein Sum2)	202.86	7.75	0.74	N.A.	Mitosis regulation	1

PAAG_01298	M protein repeat protein	185.34	27.00	***	N.A.	Mitotic spindle assembly checkpoint	2
TRANSCRIPTION							
RNA synthesis							
PAAG_08382	Lamina-associated polypeptide 2	389.94	7.00	***	N.A.	Transcription regulation	2
PAAG_04496	nascent polypeptide-associated complex subunit beta	1549.00	6.33	0.54	N.A.	Transcription regulation	2
PAAG_01597	predicted protein (bZIP transcription factor)	1032.22	6.00	***	N.A.	Transcription regulation	2
PAAG_02467	conserved hypothetical protein (transcription initiation factor TFIID subunit 14)	821.26	5.50	***	N.A.	Transcription initiation	2
RNA processing							
PAAG_04662	cleavage and polyadenylation specificity factor subunit 5	269.70	3.00	***	N.A.	mRNA processing	1
PROTEIN SYNTHESIS							
Ribosome biogenesis							
PAAG_05017	40S ribosomal protein S10-A	525.66	3.00	***	N.A.	Translation	2
PAAG_08634	40S ribosomal protein S12	449.23	6.17	0.72	N.A.	Translation	2
PAAG_05704	40S ribosomal protein S13-1	1500.97	7.40	0.79	N.A.	Translation	2
PAAG_04690	40S ribosomal protein S15	7159.23	3.67	0.72	N.A.	Translation	2

PAAG_05778	40S ribosomal protein S19	2639.44	5.83	0.64	N.A.	Translation	2
PAAG_03322	40S ribosomal protein S20	831.39	3.00	0.70	N.A.	Translation	2
PAAG_05805	40S ribosomal protein S21	3021.22	4.20	0.77	N.A.	Translation	2
PAAG_06882	40S ribosomal protein S24	854.62	5.20	0.67	N.A.	Translation	2
PAAG_01785	40S ribosomal protein S3	2242.64	12.83	0.80	N.A.	Translation	2
PAAG_07707	60S ribosomal protein L10a	318.92	9.50	***	N.A.	Translation	1
PAAG_04425	60S ribosomal protein L22	3250.68	6.60	0.73	N.A.	Translation	2
PAAG_05233	60S ribosomal protein L26	1240.01	6.00	***	N.A.	Translation	2
PAAG_01050	cytosolic large ribosomal subunit protein L30	920.25	4.20	0.72	N.A.	Translation	2
PAAG_09096	hypothetical protein (40S ribosomal protein S28)	4232.84	3.17	0.74	N.A.	Translation	2
Translation							
PAAG_02024	elongation factor 1-alpha	346.65	4.00	***	N.A.	Translation elongation	2
PAAG_00241	eukaryotic translation initiation factor 5A	289.07	3.00	***	N.A.	Translation initiation	2
PAAG_00240	eukaryotic translation initiation factor 5A	538.18	6.67	0.78	N.A.	Translation initiation	2
PAAG_06623	translation initiation factor 4B	207.36	8.67	0.56	N.A.	Translation initiation	1
PAAG_00772	hypothetical protein (eukaryotic translation initiation factor 3	182.70	1.00	***	N.A.	Translation initiation	1

	subunit J)						
PAAG_05882	translation factor SUI1	907.08	3.50	***	N.A.	Translation regulation	2
Aminoacyl-tRNA-synthethases							
PAAG_08172	lysyl-tRNA synthetase	150.31	10.00	***	6.1.1.6	Aminoacyl-tRNA biosynthesis	1
PROTEIN FATE (folding, modification, destination)							
Protein folding and stabilization							
PAAG_00739	peptidyl-prolyl cis-trans isomerase B	8036.89	11.00	0.80	5.2.1.8	Protein folding	2
PAAG_07775	heat shock protein SSB1	1838.43	14.33	0.79	N.A.	Protein folding	2
PAAG_01778	peptidyl-prolyl cis-trans isomerase H	1794.67	5.33	0.73	5.2.1.8	Protein folding	2
Protein targeting, sorting and translocation							
PAAG_07890	vacuolar-sorting protein snf7	206.78	4.00	***	N.A.	Protein targeting to vacuole	1
Protein modification							
PAAG_03038	ubiquitin-like modifier SUMO	730.49	3.00	***	N.A.	Protein sumoylation	2
Assembly of protein complexes							
PAAG_01735	hypothetical protein (cytochrome c oxidase copper chaperone Cox17)	2250.75	2.50	***	N.A.	Respiratory chain complex IV assembly	2
PAAG_07543	conserved hypothetical protein (tubulin-specific chaperone Rbl2)	678.88	2.80	0.66	N.A.	Tubulin complex assembly	2

Protein/peptide degradation							
PAAG_04282	UBX domain-containing protein	197.43	9.00	***	N.A.	Proteasomal degradation	1
PAAG_07500	Xaa-Pro aminopeptidase	179.14	6.00	***	3.4.11.9	Cytoplasmic and nuclear protein degradation	2
PAAG_04168	aqualysin-1	1117.37	7.33	0.80	3.4.21.111	Proteolysis	2
PAAG_00664	aspartyl aminopeptidase	969.00	14.83	0.76	3.4.11.21	Proteolysis	2
PAAG_01095	proline iminopeptidase	310.95	4.50	0.75	3.4.11.5	Proteolysis	1
PAAG_05466	xaa-Pro dipeptidase	380.68	5.75	0.74	3.4.13.9	Proteolysis	2
PROTEIN WITH BINDING FUNCTION OR COFACTOR REQUIREMENT							
Nucleic acid binding							
PAAG_03823	conserved hypothetical protein (RRM domain-containing protein)	178.55	3.00	***	N.A.	RNA binding	1
PAAG_05720	conserved hypothetical protein (SAP domain-containing protein)	877.35	5.80	0.80	N.A.	Nucleic acid binding	2
CELLULAR TRANSPORT, TRANSPORT FACILITIES AND TRANSPORT ROUTES							
Transported compounds							
PAAG_00109	mitochondrial intermembrane space translocase subunit Tim	854.31	4.00	***	N.A.	Protein transport	2
PAAG_07386	conserved hypothetical protein (import inner membrane	384.76	1.00	***	N.A.	Protein transport	2

	translocase subunit TIM)						
Transport routes							
PAAG_06233	vesicular-fusion protein SEC17	161.40	5.00	***	N.A.	ER to Golgi transport	1
PAAG_08252	clathrin light chain	354.63	2.20	0.70	N.A.	Vesicular transport	2
PAAG_04651	GTP-binding nuclear protein GSP1/Ran	853.14	2.17	0.39	N.A.	Nuclear transport	2
PAAG_02465	hypothetical protein (ran-specific GTPase-activating protein)	179.71	2.00	***	N.A.	Nuclear transport	1
CELLULAR COMMUNICATION/SIGNAL TRANSDUCTION MECHANISM							
Cellular signalling							
PAAG_08247	calmodulin	5032.47	8.83	0.73	N.A.	Ca2+ mediated signal transduction	2
BIOGENESIS OF CELLULAR COMPONENTS							
Cell wall							
PAAG_04235	hydrophobin	11190.10	5.33	0.79	N.A.	Cell wall organization	2
CELLULAR COMMUNICATION/ SIGNAL TRANSDUCTION MECHANISM							
Transmembrane signal transduction							
PAAG_02817	stomatin family protein	192.30	5.50	***	N.A.	Ion channel mediated signalling pathway	1
INTERACTION WITH THE ENVIRONMENT							

Homeostasis							
PAAG_02622	iron-binding protein iscA	209.72	6.00	***	N.A.	Iron-sulfur cluster assembly	1
PAAG_02974	glutaredoxin domain-containing protein	181.12	3.00	***	N.A.	Cell redox homeostasis	1
SUBCELLULAR LOCALIZATION							
Nucleus							
PAAG_08118	conserved hypothetical protein	212.37	6.00	***	N.A.	Lin1 family protein	2
UNCLASSIFIED PROTEINS							
PAAG_02217	isochorismatase domain-containing protein	1462.05	5.20	0.69	N.A.	N.A.	2
PAAG_09108	RPEL repeat protein	396.89	5.00	***	N.A.	N.A.	2
PAAG_09001	conserved hypothetical protein	247.80	1.00	***	N.A.	N.A.	2
PAAG_08409	hypothetical protein	2939.15	3.00	***	N.A.	N.A.	2
PAAG_05403	predicted protein	255.76	6.00	***	N.A.	N.A.	1
PAAG_04178	conserved hypothetical protein	178.05	2.00	***	N.A.	N.A.	1
PAAG_02985	hypothetical protein	214.03	5.00	***	N.A.	N.A.	1
PAAG_02839	hypothetical protein (HMG box protein)	342.55	8.00	***	N.A.	N.A.	2
PAAG_02001	conserved hypothetical protein	880.54	4.00	***	N.A.	N.A.	2

PAAG_01567	conserved hypothetical protein	690.12	5.00	***	N.A.	N.A.	2
PAAG_00467	predicted protein	238.33	4.00	***	N.A.	N.A.	1
PAAG_00026	conserved hypothetical protein	328.98	6.00	***	N.A.	N.A.	1
PAAG_00233	conserved hypothetical protein (rhodanese domain-containing protein)	462.00	1.00	***	N.A.	N.A.	2
PAAG_08015	hypothetical protein	1113.98	1.00	0.80	N.A.	N.A.	2
PAAG_07772	conserved hypothetical protein	1321.21	7.50	0.74	N.A.	N.A.	2
PAAG_04083	conserved hypothetical protein (isochorismatase family hydrolase)	1368.58	3.67	0.69	N.A.	N.A.	2
PAAG_00297	conserved hypothetical protein (F1F0-ATP synthase regulatory factor Stf2)	561.22	4.25	0.59	N.A.	N.A.	2

[a]Information obtained from *Paracoccidioides* Database (http://www.broadinstitute.org/annotation/genome/paracoccidioides_brasiliensis/MultiHome.html)
[b]filter 1 – proteins derived from PepFrag2; filter 2 – proteins derived from PepFrag1, as determined by PLGS and cited by Murad and Rech (2012).
***: proteins identified just in presence of inorganic iron;
N.A.: not applicable.

III. DISCUSSÃO

Os macrófagos alveolares são as primeiras células de defesa que interagem com *Paracoccidioides* spp. A ativação desses fagócitos é fundamental para o controle do crescimento fúngico (LOURES et al., 2010). Já foi demonstrado que macrófagos residentes ou monócitos, não ativados por citocinas, são permissivos à multiplicação intracelular de *Paracoccidioides* spp. Nesse caso, essas células fagocíticas representariam um ambiente protetor contra o sistema complemento, anticorpos ou componentes da resposta imune inata para o fungo, permitindo a disseminação do micro-organismo para diferentes órgão e tecidos (BRUMMER et al., 1989; MOSCARDI-BACCHI; BRUMMER; STEVENS, 1994). Além disso, o fungo pode se disseminar pelo corpo humano através da via hematogênica (FRANCO, 1987). Dessa maneira, *Paracoccidioides* spp. tem pelo menos duas oportunidades de entrar em contato com a hemoglobina: (1) dentro dos macrófagos, que já foram apontados como células recicladoras de hemácias senescentes (HENTZE et al., 2010); (2) e durante a disseminação pelo sangue. É comum acontecer a lise intravascular de hemácias, principalmente senescentes. Neste momento, os micro-organismos têm que competir pela hemoglobina ou grupo heme livres com a haptoglobina e a hemopexina, glicoproteínas do hospedeiro capazes de se ligar, respectivamente, a essas moléculas que contêm ferro (HENTZE et al., 2010; CASSAT; SKAAR, 2013).

Dessa maneira, a hemoglobina e o grupo heme poderiam representar fontes de ferro importantes para *Paracoccidioides* spp. durante a infecção do hospedeiro. Além disso, já foi demonstrado que o sangue humano não é um ambiente ferro limitante para o fungo (BAILÃO et al., 2006), o que também sugere que o fungo pode utilizar hemoglobina ou grupo heme livres.Essa hipótese foi corroborada em ensaios de crescimento do fungo em meio BHI sólido suplementado com diferentes fontes de ferro (inorgânica e orgânicas). Pôde-se perceber um robusto crescimento do fungo na presença de hemoglobina e hemina. Além disso, o fungo foi capaz de internalizar o composto auto fluorescente protoporfirina IX ligada a zinco (Zn-PPIX). O anel de protoporfirina IX é intrinsicamente fluorescente, porém o ferro quela essa fluorescência, enquanto o zinco, não. Dessa forma, o composto Zn-PPIX é fluorescente e o grupo heme, não (NEVITT; THIELE, 2011). Porém, para conseguir acessar eficientemente a hemoglobina durante a infecção, *Paracoccidioides* spp. deveria apresentar potencial hemolítico. Esse potencial foi testado na presença de hemácias de carneiro. Foi demonstrado que as linhagens *Pb*18 e *Pb*01 são capazes de lisar as hemácias, reforçando a habilidade do fungo em usar essa fonte de ferro do hospedeiro.

Buscou-se no genoma do fungo por sequências homólogas às proteínas pertencentes à família de receptores de hemoglobina de *C. albicans* (WEISSMAN; KORNITZER, 2004). No genoma da linhagem *Pb*01 foram encontrados três ortólogos (Rbt5, Wap1/Csa1 e Csa2), em *Pb*18 foi encontrado apenas um ortólogo (Wap1/Csa1) e em *Pb*03 foram encontrados dois ortólogos (Rbt51 e Csa2), segundo análises *in silico*. Isso sugere que, assim como em *C. albicans* (WEISSMAN; KORNITZER, 2004) e em *C. neoformans* (CADIEUX et al., 2013), a aquisição de hemoglobina por *Paracoccidioides* spp. seja receptor-dependente. Em *C. albicans*, os transcritos *rbt5* e *wap1* são ativados em condição de baixa concentração de ferro (10 μM), quando comparada com uma condição de alta concentração de ferro (100 μM) (LAN et al., 2004). Isso pôde ser observado também para os transcritos *rbt5*, *wap1/csa1* e *csa2* de*Pb*01. Além disso, esses transcritos foram induzidos quando *Pb*01 foi cultivado em baixas concentrações de ferro (10 μM) ou na presença de hemoglobina (10 μM), quando comparado com o fungo cultivado em condições de não adição de ferro. Isso indica que as proteínas codificadas por esses transcritos são importantes durante a captação de ferro, inclusive da hemoglobina, por este fungo.

Em estudos proteômicos com a linhagem *Pb*01, Csa2 foi detectada apenas quando o fungo foi cultivado na presença de hemoglobina, sugerindo que Csa2 possa participar da aquisição dessa molécula pelo fungo. Rbt5 e Wap1/Csa1 não foram detectadas neste estudo, provavelmente porque essas proteínas são GPI-ancoradas e não foi utilizado nenhum procedimento para purificar especificamente as proteínas GPI-ancoradas à superfície do fungo. O proteoma de *Pb*01 na presença de hemoglobina também revelou que proteínas envolvidas com o metabolismo de aminoácidos, de nitrogênio e de enxofre e com a montagem de grupos Fe-S foram induzidas em comparação com o proteoma do fungo cultivado na presença de ferro inorgânico. Além disso, proteínas envolvidas com a biossíntese de porfirina foram detectadas exclusivamente quando o fungo foi cultivado na presença de ferro inorgânico. Esses resultados sugerem que *Paracoccidioides* spp. possa utilizar hemoglobina não só como fonte de ferro, mas também de nitrogênio, enxofre e porfirina, internalizando toda a molécula de hemoglobina e não somente o ferro. Essa hipótese pode ser corroborada pela identificação de proteínas envolvidas com a degradação proteica no lisossomo e no vacúolo apenas quando o fungo foi cultivado na presença de hemoglobina. Mecanismos similares já foram propostos para *C. albicans* e *C. neoformans*. Em *C. albicans*, Rbt5 ou Rbt51 liga-se à hemoglobina e o complexo é endocitado (WEISSMAN et al., 2008). Em *C. neoformans*, Cig1, uma manoproteína extracelular recentemente descrita, parece funcionar como um hemóforo ou receptor na superfície do fungo (CADIEUX et al., 2013), ajudando na captação de hemoglobina pelo fungo, provavelmente por endocitose (HU et al., 2013).

Como observado, *Pb*01 *rbt5* apresentou o maior nível de regulação na presença de hemoglobina. Por isso, a proteína Rbt5 foi investigada neste trabalho. Para isso, o transcrito codificante para *Pb*01 Rbt5 foi clonado em um vetor de expressão e a proteína recombinante foi produzida fusionada à GST em *Escherichia coli*. Após purificação em resina de afinidade por GST, esta proteína foi clivada e a Rbt5 recombinante foi obtida e utilizada para a produção de anticorpos policlonais em coelho.*Pb*01 Rbt5 demonstrou possuir características semelhantes à Rbt5 de *C. albicans*, como domínio CFEM (ALMEIDA; WILSON; HUBE, 2009) e âncora GPI (DE GROOT; HELLINGWERF; KLIS, 2003). Além disso, a proteína foi identificada na superfície de leveduras de *Pb*01, onde provavelmente se encontre GPI-ancorada. A capacidade da ligação da proteína recombinante a hemina, protoporfirina e hemoglobina também foi investigada, demonstrando que *Pb*01 Rbt5 é capaz de se ligar a todas essas moléculas. Esses dados reforçam que *Pb*01 Rbt5 deve participar da captação de hemoglobina em *Paracoccidioides* spp.

Para investigar o papel funcional de *Pb*01 Rbt5, uma linhagem silenciada para o gene codificante desta proteína foi construída. O silenciamento foi realizado utilizando a tecnologia de RNA antisentido (aRNA) juntamente com a transformação mediada por *A. tumefasciens* (ATMT), como já descrito (ALMEIDA et al., 2009; RUIZ et al., 2011; TORRES et al., 2013). O silenciamento tanto a nível transcricional quanto a nível proteico foi demonstrado neste trabalhando utilizando PCR quantitativa em tempo real e citometria de fluxo, respectivamente. As linhagens controle utilizadas foram a selvagem e a transformada com o vetor vazio. Apesar de ter sido demonstrada a eficiência do silenciamento, não houve redução do crescimento da linhagem silenciada na presença de hemoglobina. Contrariamente, em *C. albicans* a linhagem mutante *Δrbt5* apresenta crescimento reduzido na presença de hemoglobina e hemina como fontes de ferro (WEISSMAN; KORNITZER, 2004). Esse resultado indica que baixos níveis de Rbt5 na superfície de *Paracoccidioides* spp. já seja suficiente para captar a hemoglobina ou que outros possíveis receptores de hemoglobina de *Paracoccidioides* spp. possam atuar na superfície do fungo para captar esta fonte de ferro, o que será alvo de futuros estudos em nosso laboratório.

Já foi demonstrado que o elemento ferro é importante para a sobrevivência de *Paracoccidioides* sp. dentro de monócitos (DIAS-MELICIO et al., 2006). Dessa forma, foi investigada a capacidade de a linhagem do fungo silenciada para *rbt5* sobreviver dentro de macrófagos. Foi observada uma menor sobrevida da linhagem silenciada, quando comparada às linhagens controle, dentro dos macrófagos. Além disso, a capacidade da linhagem silenciada de sobreviver dentro do tecido do hospedeiro após a infecção foi investigada utilizando um modelo murino. Foi observada uma menor carga fúngica da linhagem silenciada, em comparação às

linhagens controle, no baço de camundongos. Esses resultados indicam que Rbt5 pode atuar como um fator de virulência, contribuindo para o estabelecimento ou a manutenção da infecção, ou que esta proteína interfere na estimulação da resposta imunológica dos macrófagos para matar as células fúngicas internalizadas. Contrariamente, em *C. albicans* a deleção de *rbt5* não afetou a virulência do fungo em modelos animais de infecção (BRAUN et al., 2000), sugerindo que mecanismos compensatórios podem atuar nesse fungo na ausência de Rbt5 (WEISSMAN; KORNITZER, 2004).A habilidade de Rbt5 em atuar como um antígeno na superfície de *Paracoccidioides* spp. também foi investigada neste trabalho. Foi demonstrado que o soro de pacientes com PCM foi capaz de reconhecer a proteína recombinante Rbt5 de *Pb*01. Resultados similares foram obtidos com receptores de hemoglobina de *C. albicans*, uma vez que Rbt5 e Csa1 foram encontradas entre 33 antígenos reconhecidos por soro de pacientes convalescentes de candidemia (MOCHON et al., 2010). Esses resultados reforçam que Rbt5 pode atuar na interface entre o parasito e o hospedeiro.

Proteínas da superfície de fungos que atuam na captação de ferro podem ser alvos interessantes para o desenvolvimento de vacinas e de fármacos que bloqueiem a internalização do micronutriente e, portanto, a proliferação do micro-organismo. Além disso, essas proteínas podem servir de rota para a introdução de drogas antifúngicas dentro das células dos micro-organismos (KRONSTAD; CADIEUX; JUNG, 2013). Neste trabalho, foi demonstrado que *Paracoccidioides* spp. pode captar o grupo heme, provavelmente através de mecanismos dependentes de receptor. Dessa maneira, Rbt5 pode ser um importante alvo para o desenvolvimento de vacinas ou de fármacos bloqueadores da proliferação do patógeno, ou ainda, servir como rota para introdução de drogas antifúngicas em um estratégia do tipo cavalo de Tróia.

Capítulo 3

Homeostase de ferro

I. INTRODUÇÃO

Assim como a captação de grupo heme mediada por receptor, a captação de ferro pela via redutiva é importante para a aquisição desse micronutriente em fungos, tanto patogênicos, como *C. albicans* (KNIGHT et al., 2002) e *C. neoformans* (JUNG; DO, 2013), quanto não patogênicos, como *S. cerevisiae* (KOSMAN, 2003). O Fe^{3+} é geralmente insolúvel em pH fisiológico, na presença de oxigênio. Dessa forma, a redução deste íon para Fe^{2+} é essencial para a aquisição deste micronutriente pelos micro-organismos (KOSMAN, 2003). Após essa redução, Fe^{2+} é internalizado por uma permease dependente de uma ferro-oxidase, permitindo a internalização de Fe^{3+} (KOSMAN, 2013).

O Fe^{3+}é reduzido normalmente por uma enzima de membrana da família Fre, que contém heme e flavina em sua estrutura (KOSMAN, 2013) e que possui domínios de redutase férrica e de ligação a NAD e/ou FAD, responsáveis pela doação de elétrons (ALMEIDA; WILSON; HUBE, 2009). Alguns fungos, como *H. capsulatum*, utilizam uma redutase férrica dependente de glutationa secretada (Ggt1). Esta redutase pode utilizar sideróforos e proteínas ligantes de Fe^{3+} como substratos, sendo sua atividade enzimática mais robusta sob condições de depleção de ferro, o que é consistente com o sistema de captação de ferro de alta afinidade (ZARNOWSKI et al., 2008; TIMMERMAN et al., 2001). Além da enzima de superfície, *C. neoformans* utiliza um composto secretado chamado de ácido 3-hidroxiantranílico e a melanina para promover a redução do ferro (NYHUS; WILBORN; JACOBSON, 1997). Essa primeira etapa da via redutiva é considerada importante porque permite a aquisição de ferro a partir de uma grande variedade de compostos, como sais orgânicos e inorgânicos, sideróforos carregados com Fe^{3+} e proteínas do hospedeiro ligantes de Fe^{3+}, utilizando-se apenas um sistema de aquisição de ferro de alta afinidade (DE LUCA; WOOD, 2000; SUTAK et al., 2008).

O Fe^{2+} produzido pela enzima Fre é geralmente oxidado a Fe^{3+} por uma multicobre oxidase da família Fet. Já foi proposto que algumas lacases de basidiomicetos podem funcionar também como ferroxidases (LIU; TEWARI; WILLIAMSON, 1999; LARRONDO et al., 2003; LARRONDO et al., 2004), mas a participação dessas moléculas na captação de ferro ainda não foi caracterizada (CANESSA; LARRONDO, 2013). A ferroxidase mais estudada é a Fet3 de *S. cerevisiae*, uma proteína altamente glicosilada que possui um domínio transmembrana e quatro sítios de ligação a cobre (HASSETT; YUAN; KOSMAN, 1998). A reação catalisada por essa enzima ocorre em um destes sítios, onde o ferro é oxidado e o oxigênio molecular é reduzido a duas

moléculas de água ($4Fe^{2+} + 4H^{+} + O_2 \rightarrow 4Fe^{3+} + 2H_2O$) (KOSMAN, 2003). Esse passo de oxidação, que parece ser redundante, é considerado necessário para conceder a especificidade de transportar apenas ferro por esse mecanismo (IBRAHIM; SPELLBERG; EDWARDS, 2008). O Fe^{3+} produzido pela ferroxidase é canalizado para uma permease de ferro de alta afinidade da família Ftr, não sendo liberado no meio antes da internalização (KWOK; SEVERANCE; KOSMAN, 2006). Esse mecanismo pode auxiliar no controle do influxo de ferro, já que ainda não foi identificado um sistema regulado de excreção de ferro (KAPLAN; KAPLAN, 2009). Apenas o ferro produzido pela Fet é substrato para a Ftr (STEARMAN et al., 1996), uma proteína contendo sete domínios transmembrana (SEVERANCE; CHAKRABORTY; KOSMAN, 2004). O processamento e a localização corretos de Fet e Ftr são interdependentes. Fet3 produzida em uma célula mutada para *ftr1* (*Δftr1*) é retida em um compartimento citoplasmático em sua forma inativa, sem adição de cobre a sua estrutura. Ftr1 produzida em uma célula *Δfet3* também não atinge a membrana plasmática (KOSMAN, 2003). Esses dados sugerem que essas proteínas formam um heterodímero ou uma ultraestrutura, em que cada oligômero é importante para a maturação e o tráfico corretos do complexo para a membrana plasmática (KOSMAN, 2003; ZIEGLER et al., 2011).

Homólogos de Fet e Ftr podem ser encontrados nos genomas de muitos fungos, como *S. cerevisiae* (SINGH et al., 2006), *C. albicans* (ZIEGLER et al., 2011), *C. neoformans* (JUNG et al., 2008) e *A. fumigatus* (SCHRETTL et al., 2004), e de algas, como *Chlamydomonas reinhardtii* (TERZULLI; KOSMAN, 2010). Porém alguns fungos não possuem homólogos para essas proteínas, como é o caso de *Aspergillus nidulans* (HAAS; EISENDLE; TURGEON, 2008) e *Coccidioides immitis* (HOEGGER et al., 2006). Os organismos estudados até agora que não possuem homólogos para *fet*, também não possuem homólogos para *ftr* (HOEGGER et al., 2006). Os fungos que não possuem uma via redutiva de assimilação de ferro funcional devem utilizar sideróforos para captar o ferro extracelular (EISENDLE et al., 2003; HAAS; EISENDLE; TURGEON, 2008). Sistemas de transporte de ferro de baixa afinidade só foram identificadas até agora em *S. cerevisiae*. As permeases envolvidas neste processo, como Fet4 e Smf1, transportam, além de Fe^{2+}, outros metais, como cobre e zinco (HAAS; EISENDLE; TURGEON, 2008).

Como o excesso de ferro é tóxico, por gerar EROs, os micro-organismos desenvolveram várias estratégias para controlar não apenas a entrada de ferro, mas também sua utilização e estocagem (XU et al., 2012). A mitocôndria é particularmente importante para a homeostase e o metabolismo de ferro por ser uma das maiores consumidoras do metal na célula e, por isso, ela contém a maquinaria necessária para transporte desse íon (RICHARDSON et al., 2010; GILLE;

REICHMANN, 2011). *S. cerevisiae* possui as proteínas Mrs3 e Mrs4 pertencentes à família de carreadores mitocondriais (MCF) e que estão envolvidas com a captação de ferro para o interior da mitocôndria (FROSCHAUER; SCHWEYEN; WIESENBERGER, 2009). Os membros dessa família geralmente estão localizados na membrana mitocondrial interna, regulando a troca de substratos entre o citosol e a matriz mitocondrial. As proteínas da MCF possuem uma estrutura com três repetições sequenciais, cada uma contendo duas regiões transmembrana (ROBINSON; KUNJI, 2006). Foi demonstrado que a superexpressão de Mrs3/4 estimula a formação de heme e a maturação de proteínas contendo grupos Fe/S; e que a deleção de *mrs3/4* leva à inibição desses eventos celulares (MUHLENHOFF et al., 2003). Dessa forma, Mrs3 e Mrs4 são importantes para a homeostase intracelular de ferro e para a biossíntese de grupos heme e Fe-S (MUHLENHOFF et al., 2003; LI; KAPLAN, 2004). Homólogos dessas proteínas já foram identificados em *C. neoformans*(JACOBSON; TROY; NYHUS, 2005) e em *C. albicans* (XU et al., 2012) e também estão envolvidos com o transporte de ferro pela mitocôndria.

Outras proteínas também participam da homeostase de ferro mitocondrial, uma delas é a Yfh1, homóloga da proteína humana frataxina (PANDOLFO; PASTORE, 2009). A função de Yfh1 e seus ortólogos ainda é tema de debate na comunidade científica. Quatro hipóteses são mais aceitas (RICHARDSON et al., 2010). (1) A proteína funcionaria como um estoque mitocondrial de ferro não reativo, semelhante à ferritina, impedindo a formação de EROs, que inativariam proteínas contendo grupos Fe-S (ADAMEC et al., 2000; MORENO-CERMENO et al., 2010). (2) A Yfh1 poderia atuar como uma chaperona de ferro, doando este elemento para vias de biossíntese de grupos heme e Fe-S (MUHLENHOFF et al., 2002; PARK et al., 2003). (3) Ou ainda a proteína atuaria como um sensor mitocondrial de ferro, regulando a quantidade de grupos Fe-S formados pela disponibilidade de aceptores desse cofator (ADINOLFI et al., 2009; MORENO-CERMENO et al., 2010). (4) Além disso, já foi proposto que a frataxina, ortóloga de Yfh1, permitiria uma substituição metabólica da síntese de grupos Fe-S pela síntese do cofator heme e vice-versa, dependente do nível de expressão de enzimas envolvidas com essas vias anabólicas (Isu e ferroquelatase, respectivamente) (RICHARDSON et al., 2010). Todas essas hipóteses apontam para a importância da Yfh1 e de suas ortólogas na regulação do metabolismo intracelular de ferro (MORENO-CERMENO et al., 2010; RICHARDSON et al., 2010).

Outra forma de manter a homeostase intracelular de ferro é mantendo-o dentro do vacúolo, permitindo a estocagem e a detoxificação do metal. O ferro é provavelmente estocado em sua forma férrica (Fe^{3+}) como polifosfato (HAAS; EISENDLE; TURGEON, 2008). Os organismos controlam

rigidamente a concentração de ferro citosólico por meio da regulação da captação e da estocagem (VAN HO; WARD; KAPLAN, 2002). Em *S. cerevisiae*, Ccc1p é um transportador de Fe^{2+}/Mn^{2+} vacuolar, envolvido na transferência desses metais do citosol para o vacúolo, desempenhando um importante papel na estocagem de ferro e manganês (LI et al., 2001). O efluxo de ferro de dentro do vacúolo é realizado por Smf3, parálogo de Smf1, e por um complexo proteico formado por Fet5 e Fth1, similar ao complexo Fet3-Ftr1 da membrana plasmática (URBANOWSKI; PIPER, 1999; PORTNOY; LIU; CULOTTA, 2000). A redutase férrica Fre6, localizada na membrana do vacúolo, fornece Fe^{2+} para esses dois sistemas de efluxo vacuolares (SINGH; KAUR; KOSMAN, 2007). Alguns fungos, como *A. nidulans* e *Schizosaccharomyces pombe*, não possuem ortólogos para Fet5 e Fth1, o que sugere que esses organismos não utilizam esse sistema de estocagem de ferro, podendo utilizar sideróforos intracelulares para essa função (HAAS; EISENDLE; TURGEON, 2008).

Pouco se sabe sobre sistemas de captação e homeostase de ferro em *Paracoccidioides* spp. A atividade de uma redutase férrica extracelular dependente de glutationa já foi reportado para este fungo(ZARNOWSKI; WOODS, 2005).Além disso, já foi sugerido que o fungo utilize um transportador de ferro e zinco (Zrt) para captar o ferro extracelular, após a redução do metal por uma enzima da família Fre (BAILÃO et al., 2006; BAILÃO et al., 2007). Esses trabalhos sugerem que *Paracoccidioides* spp. possua uma via redutiva funcional para a captação do ferro extracelular, porém mais estudos precisam ser realizados para se entender melhor os mecanismos moleculares envolvidos com a aquisição e a homeostase deste metal.

II. ARTIGO PUBLICADO NA REVISTA FRONTIERS IN MICROBIOLOGY

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The homeostasis of iron, copper, and zinc in *Paracoccidioides brasiliensis*, *Cryptococcus neoformans* var. *grubii*, and *Cryptococcus gattii*: a comparative analysis

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Iron, copper, and zinc are essential for all living organisms. Moreover, the homeostasis of these metals is vital to microorganisms during pathogenic interactions with a host. Most pathogens have developed specific mechanisms for the uptake of micronutrients from their hosts in order to counteract the low availability of essential ions in infected tissues. We report here an analysis of genes potentially involved in iron, copper, and zinc uptake and homeostasis in the fungal pathogens *Paracoccidioides brasiliensis*, *Cryptococcus neoformans* var. *grubii*, and *Cryptococcus gattii*. Although prior studies have identified certain aspects of metal regulation in *Cryptococcus* species, little is known regarding the regulation of these elements in *P. brasiliensis*. We also present amino acid sequences analyses of deduced proteins in order to examine possible conserved domains. The genomic data reveals, for the first time, genes associated to iron, copper, and zinc assimilation and homeostasis in *P. brasiliensis*. Furthermore, analyses of the three fungal species identified homologs to genes associated with high-affinity uptake systems, vacuolar and mitochondrial iron storage, copper uptake and reduction, and zinc assimilation. However, homologs to genes involved in siderophore production were only found in *P. brasiliensis*. Interestingly, *in silico* analysis of the genomes of *P. brasiliensis Pb*01, *Pb*03, and *Pb*18 revealed significant differences in the presence and/or number of genes involved in metal homeostasis, such as in genes related to iron reduction and oxidation. The broad analyses of the genomes of *P. brasiliensis, C. neoformans* var. *grubii*, and *C. gattii* for genes involved in metal homeostasis provide important groundwork for numerous interesting future areas of investigation that are required in order to validate and explore the function of the identified genes and gene pathways.

Keywords: micronutrient homeostasis, pathogenic fungi, infection

INTRODUCTION

A sufficient supply of iron, copper and zinc is essential for all living and proliferating organisms. In infectious diseases, iron, copper and zinc metabolism are important for both the host and the pathogen, and complex responses in each occur to maintain adequate resources of these elements to preserve homeostasis. Iron, in the form of heme and iron–sulfur clusters, is essential as a cofactor of various enzymes, oxygen carriers, and electron-transfer systems involved in vital cellular functions ranging from respiration to DNA replication (Schaible and Kaufmann, 2004). Copper is a redox-active metal ion essential for most aerobic organisms, which also serves as a catalytic and structural cofactor for enzymes involved in energy generation, iron acquisition, oxygen transport, and cellular metabolism, among other processes (Kim et al., 2008). Zinc is also a crucial metal, since it is at the catalytic center of numerous enzymes and plays important roles in the functionality of a wide variety of proteins (Van Ho et al., 2002). Mammalian hosts and microbes have developed sophisticated strategies to acquire these metals, even under conditions in which their availability is limited. One of the strategies developed by mammalian hosts to prevent microbial infections is to limit the availability of iron (Weinberg, 2009). Recently, it has been demonstrated that zinc deprivation is a host defense mechanism utilized by macrophages during *Histoplasma capsulatum* infection (Winters et al., 2010). In addition, the binding of copper to calgranulin C in human neutrophils could be a mechanism of antimicrobial action (Moroz et al., 2003). In order to counteract these and other host responses, microorganisms employ a range of uptake mechanisms for the targeted acquisition of iron, copper and zinc.

Ferric iron is generally insoluble at physiological pH in the presence of oxygen. Thus, the common mechanisms of iron-assimilation include the reduction of ferric (Fe^{3+}) to ferrous (Fe^{2+}),

and solubilization of Fe^{3+} by binding siderophores (Kornitzer, 2009). The reductive system in fungi is regulated by three different mechanisms. First, a low-affinity iron reductase that functions in iron-rich environments generates Fe^{2+}, which is transported into the cell by a non-specific low-affinity iron permease. Second, a regulated high-affinity ferric reductase operates in low iron conditions, such as those present in a mammalian host. The produced Fe^{2+} is further oxidized to Fe^{3+} by a membrane multi-copper-oxidase before being transported across the cell membrane by a high-affinity iron permease. The third mechanism is a non-enzymatic reduction, such as that promoted by 3-hydroxyanthranilic acid (3HAA), which is known to maintain a reduced environment to facilitate the release and sustain the presence of Fe^{2+} at the fungal membrane until transport occurs (Howard, 1999).

Ferric iron uptake mediated by siderophores is considered a non-reductive high-affinity mechanism by which microorganisms acquire iron. Siderophores are low-molecular weight (M_r < 1500), ferric iron-specific chelators (Neilands, 1993). Microorganisms produce siderophores as scavenging agents in low iron concentration environments in order to supply iron to the cell through the solubilization of extracellular ferric iron. Siderophores are also produced intracellularly for iron storage in most fungi (Matzanke et al., 1987). Siderophores can be classified into three main groups depending on the chemical nature of the moieties donating the oxygen ligands for Fe^{3+}: catechols, carboxylates and hydroxamates (Miethke and Marahiel, 2007). With the exception of the carboxylate rhizoferrin produced by zygomycetes, the other known fungal siderophores are all hydroxamates (Van der Helm and Winkelmann, 1994). Fungal hydroxamates are derived from the non-proteinogenic amino acid ornithine and can be grouped into four structural families: rhodotorulic acid, ferrichromes, coprogens and fusarinines. Siderophores are named based on their iron-charged forms, existing in the iron-free form of the ligand called desferri-siderophore. Not all fungi produce siderophores. For example, *Saccharomyces cerevisiae* is not a siderophore producer (Neilands et al., 1987). Similarly, *Cryptococcus* species and *Candida albicans* are also unable to produce siderophores. However, these pathogenic fungi can utilize iron bound to siderophores secreted by other species (bacteria and fungi), the xenosiderophores (Howard, 1999). After siderophores are synthesized, they can be utilized intracellularly or secreted to the extracellular medium to solubilize ferric iron. For secreted siderophores, the captured metal of the siderophore–iron complex may be utilized either by reductive iron assimilatory systems or by internalization of the whole complex by specific transporters. In fungi, the uptake of siderophore–iron chelates is accomplished by transporters of the siderophore–iron transporter (SIT) subfamily, previously designated as family 16 of the major facilitator superfamily (MFS; Pao et al., 1998). These transporters are integral membrane proteins, with 12–14 predicted transmembrane domains, that mediate the import of siderophores in a highly regulated process (Philpott, 2006).

Several homeostatic mechanisms that ensure the maintenance of copper at a sufficient concentration for cell growth have been identified. Copper homeostasis in fungi is maintained by the transcriptional regulation of genes involved in copper acquisition, mobilization and sequestration and also at the posttranslational level (Gross et al., 2000). In *S. cerevisiae* copper is reduced from Cu (II) to Cu (I) by cell surface metalloreductases (Hassett and Kosman, 1995; Georgatsou et al., 1997) and uptake is mediated by Ctr1p and Ctr3p, two high-affinity transporters. Both *ctr1* and *ctr3* genes are regulated at the transcriptional level in response to copper availability, being induced by copper deprivation (Dancis et al., 1994a; Pena et al., 2000). The vacuolar copper transporter Ctr2p is also involved in the intracellular copper homeostasis, since it provides copper via mobilization of intracellular copper stores (Rees et al., 2004).

Zinc homeostasis is maintained by posttranslational and transcriptional homeostatic regulatory mechanisms (Lyons et al., 2000; Eide, 2003). Unlike iron and copper, zinc is taken up as divalent cation. Once inside the cell, zinc is neither oxidized nor reduced (Berg and Shi, 1996). In *S. cerevisiae* the uptake of zinc is mediated by two separate systems. One system has a high-affinity for this metal and is active in zinc-limited conditions (Zhao and Eide, 1996a). The second system has a lower affinity for zinc and is not highly regulated by zinc concentrations (Zhao and Eide, 1996b). The expression of the high-affinity zinc transporter Zrt1p and the low-affinity zinc transporter Zrt2p is regulated by the transcription factor Zap1p, which plays a central role in zinc homeostasis (Zhao and Eide, 1997). The zinc transporter activity is also posttranslationally regulated. High levels of extracellular zinc trigger the inactivation of Zrt1p through endocytosis of the protein and its subsequent degradation in the vacuole (Gitan et al., 1998).

This paper focuses on the metabolism of iron, copper and zinc in the fungal pathogens *Paracoccidioides brasiliensis, Cryptococcus neoformans* var. *grubii*, and *Cryptococcus gattii*. Low iron conditions have been associated with the susceptibility of *P. brasiliensis*, the etiological agent of paracoccidioidomycosis (PCM), to the antimicrobial action of monocytes (Dias-Melicio et al., 2005). Major phenotypic changes in *C. neoformans*, the etiological agent of cryptococcosis, are regulated by iron availability. For example, low iron concentrations result in the induction of capsule enlargement and the repression of laccase (Jung and Kronstad, 2008). Although iron regulation is well described in *Cryptococcus* species (Jung et al., 2008), iron associated processes are poorly understood in *P. brasiliensis*. Further, there is limited information on the impact of copper and zinc in *P. brasiliensis*, as well as the impact of zinc in *Cryptococcus* species. In this paper we performed *in silico* analyses of genes related to iron, copper and zinc metabolism in *P. brasiliensis, C. neoformans* var. *grubii* and *C. gattii*. We also compared the obtained information with data available from *S. cerevisiae*, which represents the most deeply studied model fungus, and other fungi.

MATERIALS AND METHODS

Sequences of genes related to iron, copper and zinc uptake, as well as to siderophore biosynthesis and uptake were used in the search of orthologs of *P. brasiliensis* and *Cryptococcus* species genomes. The *P. brasiliensis* database[1] includes the genomes of three isolates (*Pb*01, *Pb*03, and *Pb*18) and the cryptococcal database includes genomes of *C. neoformans* var. *grubii*[2] and *C. gattii*[3]. The sequences used in

[1]http://www.broadinstitute.org/annotation/genome/paracoccidioides_brasiliensis/MultiHome.html

[2]http://www.broadinstitute.org/annotation/genome/cryptococcus_neoformans/MultiHome.html

[3]http://www.broadinstitute.org/annotation/genome/cryptococcus_neoformans_b/MultiHome.html

the *in silico* analysis were obtained from the NCBI databank[4], and they are primarily from *S. cerevisiae*, but also include genes from other fungi, such as *Aspergillus fumigatus*, *Aspergillus nidulans*, *C. albicans* and *H. capsulatum*. The search by orthologs was based on sequence similarity by using the BLAST tool. The expectation value adopted in the databases search was *E*-value $\leq 10^{-5}$.

The deduced amino acid sequences of the orthologs found in *P. brasiliensis* isolates and *Cryptococcus* species were analyzed. Searches for conserved domains and signal peptides in the orthologs proteins were performed using the Conserved Domain Database at NCBI[5] and the online software SMART[6]. Predictions of putative transmembrane segments were made using the TopPred[7] server and SMART software. Amino acid sequences alignment were performed using the ClustalX2 (Larkin et al., 2007).

RESULTS AND DISCUSSION

IRON

Uptake of iron at the cell surface by the reductive system

To better understand how *P. brasiliensis* could acquire iron by the reductive system, *in silico* analyses were performed utilizing *S. cerevisiae*[8] and *C. albicans*[9] sequences. The data showed that *Pb*01 contains four metalloreductase (Frep) homologs, *Pb*03 five homologs, and *Pb*18 three homologs (**Table 1**). The genes encoding metalloreductases were *fre1*, *fre3*, *fre5*, *fre7* and *frp1*. Also, *Pb*01 and *Pb*03 have two homologs each of the ferroxidase Fetp and *Pb*18 has one. The reductive uptake system was first described in *S. cerevisiae* (Lesuisse et al., 1987). The enzymatic reduction step in *S. cerevisiae* is catalyzed by members of the FRE family of metalloreductases. The products of the *fre* genes are not specific for iron reduction, since they can also promote copper reduction. *S. cerevisiae* Fre1p and Fre2p are required for growth on media with low concentrations of ferric iron salts. Fre3p and Fre4p catalyze uptake of iron from siderophores and Fre7p is under the control of the copper-dependent transcription factor Mac1p (Philpott and Protchenko, 2008). The expression of *C. albicans* ferric reductase Frp1p is upregulated by alkaline pH and iron-limited conditions (Liang et al., 2009). Future studies are required to dissect the roles of the different *P. brasiliensis* reductases, especially in *in vivo* conditions.

Homologs for iron permeases (Ftrp and Fthp) were not found in *P. brasiliensis* genomes, corroborating the hypothesis that iron is transported by the zinc permeases, as previously suggested by transcriptional analyses (Bailão et al., 2006, 2007; Costa et al., 2007). However, in the present *in silico* analysis, we identified five zinc transporters (**Table 1**). These permeases could be coupled with one or more of the ferroxidases homologs (Fet5p, Fet31p and Fet33p) identified in the *P. brasiliensis* genome database. In *S. cerevisiae*, reduced iron is taken up through a high-affinity transport complex that consists of Fet3p, a multi-copper ferroxidase, and Ftr1p, a permease. Independent studies have demonstrated that Fet3p produced by *S. cerevisiae* Δ*ftr1* mutant cells is retained in a cytoplasmic compartment in a copper-free, inactive form. Correspondingly, Ftr1p produced by *S. cerevisiae* Δ*fet3* mutant cells fails to reach the plasma membrane (Stearman et al., 1996). These observations are in agreement with a model in which the two proteins form a heterodimer or higher order structure for correct maturation and trafficking to the plasma membrane (Kosman, 2003).

The *P. brasiliensis* genomes analysis revealed the presence of a *ggt1* homolog. This gene is presumably responsible for the glutathione (GSH)-dependent iron reduction activity previously identified in functional studies (Zarnowski and Woods, 2005). The proposed mechanism comprises secretion of a glutathione-dependent ferric reductase (GSH–FeR), named Ggt1p, that purportedly utilizes siderophores and Fe^{3+}-binding proteins as substrates, enhancing the enzymatic activity under iron-limiting conditions, which is consistent with the function of a high-affinity uptake system, as described in *H. capsulatum* (Timmerman and Woods, 2001).

Homologs of permease genes involved in low-affinity iron reductive systems, such as *smf*, were not detected in our analysis. Hence, the low-affinity permease utilized by *P. brasiliensis* to acquire iron could be one of the zinc permeases, as suggested (**Table 1**). Despite the absence of iron permease *fth1* gene homologs, *P. brasiliensis* has one *ccc1* gene homolog that could drive iron vacuolar transport. *P. brasiliensis* also has homologs of the mitochondrial iron transporters genes *mrs3* and *mrs4* and the mitochondrial iron chaperone Yfh1p, suggesting mitochondrial iron homeostasis in this pathogen (**Table 1**). Since mitochondria are major users of iron, it follows that they should contain machinery required for its transport. Mrs3p and Mrs4p are homologous and functionally redundant proteins found in the inner mitochondrial membrane of *S. cerevisiae*, which are involved in transport under iron-limiting conditions (Foury and Roganti, 2002). Yfh1p, a homolog of human frataxin, is also involved in mitochondrial iron homeostasis (Babcock et al., 1997). While Mrs3p and Mrs4p mediate iron delivery from the outside to the inside of mitochondria, the frataxin homolog facilitates the use of iron within this organelle, functioning as a mitochondrial matrix iron chaperone (Zhang et al., 2006; Froschauer et al., 2009).

Cryptococcal genomic databases analysis revealed both *S. cerevisiae* and *C. albicans* homologs for proteins related to iron metabolism (**Table 1**). Remarkably, the *C. neoformans* var. *grubii* database contains four metalloreductase homologs, while the *C. gattii* genome has three similar homologs. The reason for the multiplicity of metalloreductases isoenzymes is not clear, although it is speculated that some sets of genes are expressed under specific conditions for iron acquisition (Kornitzer, 2009). Concerning the ferroxidases, *C. neoformans* var. *grubii* has three homologs and *C. gatti* contains one. Both genomes possess two iron permeases homologs, whose presence is supported by prior functional analyses (Jung et al., 2008). Two iron permeases, gene orthologs of *S. cerevisiae ftr1*, have been identified in *C. neoformans,* namely Cft1p and Cft2p (Jung et al., 2008). The expression of the *cft1* gene is down-regulated at high iron concentrations, suggesting that its product functions as a high-affinity iron permease. The role of *cft2* is still unclear, although it supposedly encodes a low-affinity iron permease or a vacuolar permease that could transport stored iron to the cytoplasm, similar to what occurs in *S. cerevisiae* with the iron permease Fth1p. One of the iron permeases here identified is probably a Fth1p homolog, which

[4]http://www.ncbi.nlm.nih.gov/guide/

[5]http://www.ncbi.nlm.nih.gov/Structure/cdd/wrpsb.cgi

[6]http://smart.embl-heidelberg.de/

[7]http://mobyle.pasteur.fr/cgi-bin/portal.py?form=toppred

[8]http://www.yeastgenome.org/

[9]http://www.candidagenome.org/

Table 1 | Orthologs to genes related to iron, copper and zinc uptake by reductive systems in *P. brasiliensis* and *Cryptococcus* species.

Gene	Organism/accession number	Predicted function	Orthologs in *Pb* 01, 03 and 18 (accession numbers)†	*E*-value*	Orthologs in *Cryptococcus* species (accession numbers)†	*E*-value*
fre1	*S. cerevisiae* NP_013315	Metalloreductase	PAAG_05370.1 PABG_06003.1	e-22 e-19	Not identified	–
fre3	*S. cerevisiae* NP_015026	Metalloreductase	PAAG_02079.1 PABG_02329.1 PADG_00813.1	e-35 e-35 e-35	Not identified	–
fre5	*S. cerevisiae* NP_015029	Metalloreductase	PABG_07812.1	e-26	Not identified	–
fre7	*S. cerevisiae* NP_014489	Metalloreductase	PAAG_06164.1 PABG_06497.1 PADG_07957.1	0.0 0.0 0.0	CNAG_00876.2 CNBG_6082.2	e-37 e-37
fre8	*S. cerevisiae* NP_013148	Metalloreductase	Not identified	–	CNAG_07334.2 CNBG_2116.2	e-10 e-07
fre10	*C. albicans* XP_711543	Metalloreductase	Not identified	–	CNAG_06821.2 CNBG_5888.2	e-34 e-27
cfl4	*C. albicans* XP_715639	Metalloreductase	Not identified	–	CNAG_06524.2	e-32
frp1	*C. albicans* XP_713315	Metalloreductase	PAAG_04493.1 PABG_04278.1 PADG_04652.1	e-26 e-26 e-26	Not identified	–
fet3	*S. cerevisiae* NP_013774	Ferroxidase	Not identified	–	CNAG_06241.2	0.0
fet5	*S. cerevisiae* NP_116612	Ferroxidase	PABG_05667.1 PADG_05994.1	e-40 e-37	CNAG_07865.2 CNBG_4942.2	0.0 0.0
fet31	*C. albicans* XP_711263	Ferroxidase	PAAG_06004.1	e-39	CNAG_02958.2	0.0
fet33	*C. albicans* XP_711265	Ferroxidase	PAAG_00163.1 PABG_05183.1	e-33 e-33	Not identified	–
ftr1/ftr2	*C. albicans* XP_715020/ XP_715031	Iron permease	Not identified	–	CNAG_06242.2 CNBG_3602.2	0.0 0.0
smf1	*S. cerevisiae* NP_014519	Low-affinity permease	Not identified	–	CNAG_05640.2 CNBG_6162.2	0.0 0.0
fth1	*C. albicans* XP_723298	Vacuolar transporter	Not identified	–	CNAG_02959.2 CNBG_4943.2	0.0 0.0
ccc1	*S. cerevisiae* NP_013321	Vacuolar transporter	PAAG_07762.1 PABG_00362.1 PADG_02775.1	e-31 e-31 e-31	CNAG_05154.2 CNBG_4540.2	e-23 e-18
mrs3/ mrs4	*S. cerevisiae* NP_012402/ NP_012978	Mitochondrial iron transporter	PAAG_05053.1 PABG_04509.1 PADG_04903.1	0.0 0.0 0.0	CNAG_02522.2 CNBG_4218.2	0.0 0.0
yfh1	*S. cerevisiae* NP_010163	Mitochondrial matrix iron chaperone	PAAG_02608.1 PABG_03095.1 PADG_01626.1	e-15 e-09 e-16	CNAG_05011.2 CNBG_4670.2	e-18 e-18

(Continued)

Table 1 | Continued

Gene	Organism/accession number	Predicted function	Orthologs in *Pb* 01, 03 and 18 (accession numbers)†	*E*-value*	Orthologs in *Cryptococcus* species (accession numbers)†	*E*-value*
ggt1	*H. capsulatum* EGC49121	Secreted glutathione-dependent ferric reductase	PAAG_06130.1 PABG_06527.1 PADG_07986.1	0.0 0.0 0.0	CNAG_02888.2 CNBG_3537.2	0.0 0.0
mac1	*S. cerevisiae* NP_013734	Copper metalloregulatory transcription factor	PAAG_08210.1 PABG_07429.1	e-5 e-5	CNAG_07724.2 CNBG_2252.2	e-7 e-7
ctr3	*S. cerevisiae* NP_013515	High-affinity copper transporter of the plasma membrane	PAAG_05251.1 PABG_07607.1 PADG_05084.1	e-22 e-21 e-21	CNAG_00979.2 CNBG_0560.2	e-14 e-14
ctr1	*S. cerevisiae* NP_015449	High-affinity copper transporter of the plasma membrane	Not identified	–	Not identified	–
ctr2	*S. cerevisiae* NP_012045	Putative low-affinity copper transporter of the vacuolar membrane	PABG_01536.1 PADG_04146.1	e-14 e-14	CNAG_01872.2	e-13
atx1	*S. cerevisiae* NP_14140	Cytosolic copper metallochaperone	PAAG_00326.1 PABG_06615.1 PADG_02352.1	e-12 e-12 e-12	CNAG_02434.2 CNBG_4136.2	e-10 e-11
ccc2	*S. cerevisiae* NP_010556	Cu^{2+} transporting P-type ATPase	PAAG_07053.1 PABG_03057.1 PADG_01582.1	0.0 0.0 0.0	CNAG_06415.2 CNBG_5045.2	0.0 0.0
cup1	*S. cerevisiae* NP_011920	Metallothionein	Not identified	–	Not identified	–
cup2	*S. cerevisiae* NP_011922	Metallothionein	Not identified	–	Not identified	–
sod1	*S. cerevisiae* NP_012638	Cytosolic superoxide dismutase	PAAG_04164.1 PABG_03954.1 PADG_07418.1	0.0 0.0 0.0	CNAG_01019.2 CNBG_0599.2	0.0 0.0
sod2	*S. cerevisiae* NP_011872	Mitochondrial superoxide dismutase	PAAG_02725.1 PABG_03204.1 PADG_01755.1	0.0 0.0 0.0	CNAG_04388.2 CNBG_2661.2	0.0 0.0
*zrt*1	*S. cerevisiae* NP_011259	High-affinity zinc transporter of the plasma membrane	PAAG_08727.1 PABG_07725.1 PADG_08567.1	0.0 0.0	CNAG_03398.2 CNBG_2209.2	e-40 e-41
zrt2	*S. cerevisiae* NP_013231	Low-affinity zinc transporter of the plasma membrane	PAAG_03419.1 PABG_05498.1 PADG_06417.1	e-27 e-26 e-28	CNAG_00895.2	0.0

(Continued)

Table 1 | Continued

Gene	Organism/accession number	Predicted function	Orthologs in *Pb* 01, 03 and 18 (accession numbers)†	*E*-value*	Orthologs in *Cryptococcus* species (accession numbers)†	*E*-value*
*zrc*1	*S. cerevisiae* NP_013970	Vacuolar membrane zinc transporter	PAAG_00702.1	e-41	Not identified	–
*cot*1	*S. cerevisiae* NP_014961	Vacuolar membrane zinc transporter	PAAG_07885.1 PABG_07467.1 PADG_08196.1	e-44 0.0 0.0	CNAG_02806.2 CNBG_3460.2	e-40 e-37
*zrt*3	*S. cerevisiae* NP_012746	Vacuolar membrane zinc transporter	PAAG_09074.1 PABG_04697.1 PADG_05322.1	e-23 e-22 e-23	Not identified	–
*msc*2	*S. cerevisiae* NP_010491	Cation diffusion facilitator protein of the endoplasmic reticulum and nucleus	PABG_07115.1 PADG_06381.1	e-40 e-40	CNAG_05394.2 CNBG_4458.2	e-23 e-24
*zap*1	*S. cerevisiae* NP_012479	Zinc-regulated transcription factor	PAAG_03645.1 PABG_03305.1 PADG_01870.1	e-20 e-18 e-24	CNAG_05392.2 CNBG_4460.2	e-40 e-28

**Similarities with E-values < 10^{-5} were considered significant.*
†Accession numbers: PAAG refers to Pb01; PABG refers to Pb03; PADG refers to Pb18; CNAG refers to C. neoformans *var.* grubii *and CNBG refers to* C. gattii.

is likely involved in vacuolar iron uptake. Moreover, we could identify iron transporter *ccc1* gene homologs in the genome, suggesting that a vacuolar iron homeostasis system exists in *Cryptococcus*. Data mining revealed one homolog of the low-affinity gene *smf* family, confirming the presence of both high and low-affinity iron reductase systems, as described (Jacobson et al., 1998). The presence of mitochondrial *mrs3, mrs4* and *yfh1* gene homologs in *C. neoformans* var. *grubii* supports a mechanism for iron homeostasis (Nyhus and Jacobson, 1999; Jacobson et al., 2005). Additionally, our *in silico* analyses demonstrated that cryptococcal reductive systems are closely related to that of *S. cerevisiae* (**Table 1**). Although no activity for the enzyme glutathione-dependent ferric reductase had been reported in *Cryptococcus*, both genomes contain *ggt1* homologs suggesting the presence of a GSH–FeR system. A comparative analysis of iron uptake by reductive systems in *P. brasiliensis*, *C. neoformans* var. *grubii* and *C. gattii* is depicted in **Figure 1**.

Conserved domains in proteins related to the reductive iron metabolism

Amino acid sequence analyses of orthologs proteins found in the *P. brasiliensis* isolates and *Cryptococcus* species may support the assumption of conserved functions. Searching for conserved domains in all the analyzed sequences (**Table A1** in Appendix) revealed that most of the *P. brasiliensis* and *Cryptococcus* deduced proteins codified by the genes related to reductive iron metabolism contain conserved domains related to specific functions. Regarding to metalloreductases, the presence of a ferric reductase domain and a FAD- and/or a NAD-binding domain can be essential for functional enzymatic activity, since they are responsible for electron donation, as described in other organisms (De Luca and Wood, 2000). A schematic diagram presenting the cited motifs in a metalloreductase Frep is shown in **Figure 2**. An HPFT*XX*S motif is believed to be a site for FAD-binding and a glycine-rich motif and a cysteine–glycine couple are thought to be involved in NADPH binding (Shatwell et al., 1996). As well, copper-oxidase domains are required for ferroxidase activity. *S. cerevisiae* Fet3p is a multi-copper-oxidase and, like other copper proteins, possesses three distinct types of Cu^{2+}-binding sites. Oxidation of Fe^{2+} occurs at the type 1 copper site followed by the reduction of molecular oxygen to $2H_2O$ at the other two copper sites (Hassett et al., 1998; Kosman, 2003). The ferroxidases in the *P. brasiliensis* isolates and *Cryptococcus* species present such domain, suggesting they are functional proteins.

Siderophore production

Culture supernatants of *P. brasiliensis* grown in media with low iron concentrations display higher iron binding capacity when compared with culture supernatants from iron-rich media (Arango and Restrepo, 1988), which has suggested that siderophores are involved in iron acquisition in this fungus. Furthermore, *in silico* analysis of *P. brasiliensis* structural genomes indicates that this fungus can potentially produce siderophores. The three sequenced *P. brasiliensis* genomes show sequences that potentially encode all the necessary enzymes for siderophore synthesis: *sidA*, *sidF*, *sidC* and *sidD* (*A. fumigatus* orthologs), as shown in **Table 2** and **Figure 1**. This biosynthetic pathway may lead to the production of hydroxamate-type siderophores. The first committed step in siderophore biosynthesis is the N^5-hydroxylation of ornithine catalyzed by ornithine-N^5-oxygenase. The *sid1* gene of *Ustilago maydis,* the etiologic agent of corn smut, was the first characterized fungal ornithine-N^5-oxygenase-encoding gene (Mei et al., 1993).

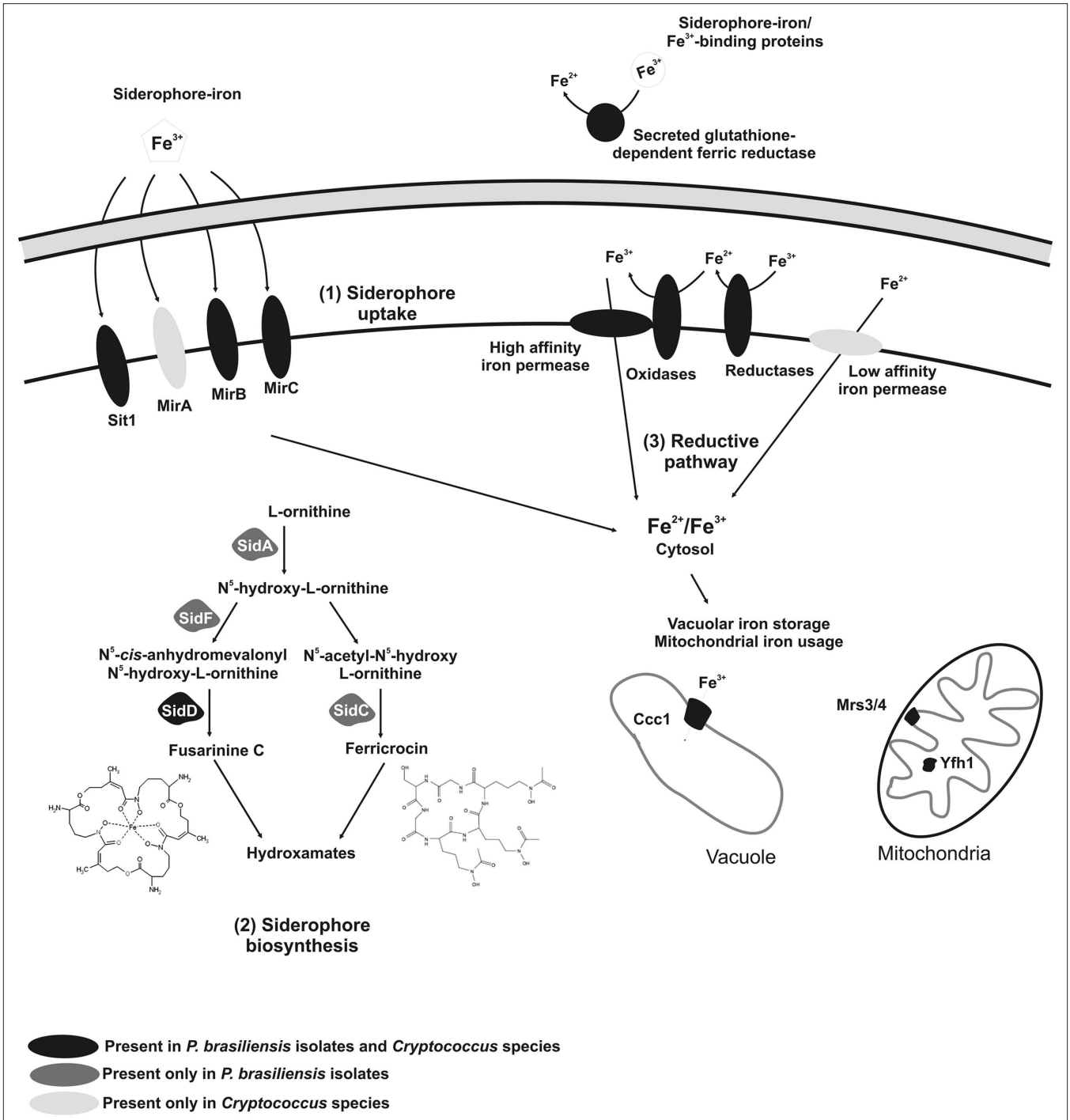

FIGURE 1 | Schematic comparison of iron metabolism in *P. brasiliensis* isolates and *Cryptococcus* species. Sit1p, MirAp, MirBp and MirCp are membrane transporters that traffic siderophores bound to ferric iron into the intracellular environment. SidAp, SidFp, SidCp and SidDp are enzymes from the biosynthetic pathway of hydroxamate-type siderophores. Ccc1p is a vacuolar membrane iron transporter. Mrs3/4p are iron transporters found in the inner mitochondrial membrane and Yfh1p is a mitochondrial matrix iron chaperone.

Orthologs of *sid1* have been identified in *A. fumigatus* (*sidA*) and *H. capsulatum* (*sid1*). In the latter, disruption of *sid1* causes poor growth under low iron conditions and loss of siderophore production, suggesting an important role of siderophore production in iron-limiting conditions (Schrettl et al., 2004; Hwang et al., 2008). The formation of the hydroxamate group consists of the transfer of an acyl group from acyl-coenzyme A to N^5-hydroxyornithine. Different acyl group usage results in the production of distinct

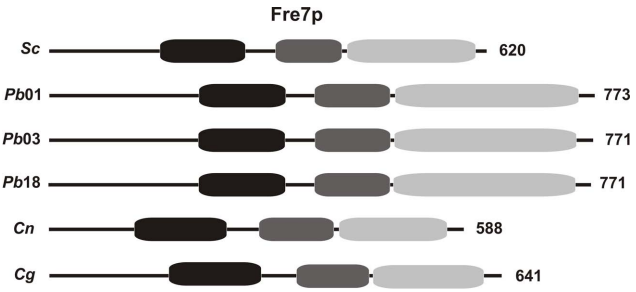

FIGURE 2 | Domains found in a Frep related to iron reductive uptake in *P. brasiliensis* isolates and *Cryptococcus* species. The found domains are: ferric reductase domain (black boxes), FAD-binding domain (dark gray boxes) and NAD-binding domain (light gray boxes). The length of each protein, in amino acids, is shown on the right. Accession numbers: *Sc* (NP_014489), *Pb*01 (PAAG_06164.1), *Pb*03 (PABG_06497.1), *Pb*18 (PADG_07957.1), *Cn* (CNAG_00876.2) and *Cg* (CNBG_6082.2).

siderophores. Acetyl is used for rhodotorulic acid and ferrichrome synthesis, while anhydromevalonyl is utilized in the fusarinines and coprogens pathway (Haas et al., 2008). *A. fumigatus sidF* encodes an N^5-hydroxyornithine:*cis* anhydromevalonyl coenzyme A-N^5-transacylase involved in the synthesis of fusarinine and triacetylfusarinine (Schrettl et al., 2007). The *sidF* ortholog of *H. capsulatum, sid3* gene, is transcriptionally induced under iron restricted conditions (Hwang et al., 2008). Hydroxamates are covalently linked via peptide (rhodotorulic acid, ferrichromes, coprogens) or ester bonds (fusarinines, coprogens) carried out by non-ribosomal peptide synthetases (NRPSs; Finking and Marahiel, 2004). In *A. fumigatus*, *sidC* and *sidD* encode two NRPSs involved in ferricrocin (intracellular siderophore) and triacetylfusarinine C (TAFC) biosynthesis, respectively. Some siderophores additionally require acetylation at the N^2-amino group, such as coprogen and TAFC. For example, *sidG* deletion in *A. fumigatus* results in the abrogation of the TAFC siderophore production (Schrettl et al., 2007). Given that our *in silico* analysis of *P. brasiliensis* identified sequences capable of coding for SidAp, SidFp, SidCp and SidDp, it is reasonable to hypothesize that *P. brasiliensis* may be able to synthesize both extracellular and intracellular siderophores.

Although *Cryptococcus* species have been described as unable to produce siderophores (Jacobson and Petro, 1987), *in silico* analysis of *C. neoformans* var. *grubii* and *C. gattii* structural genomes indicates the presence of *sidD* and *sidG* genes, which are also involved in other metabolic pathways in fungi. However, *sidA* and *sidF* genes were not found, and these genes are essential, especially since they act early in the pathway for siderophores production (**Table 2**; **Figure 1**). It will be interesting to examine if *sidA* and *sidF* have other functions and how siderophore-associated iron uptake was replaced to account for this loss.

Conserved domains in proteins related to siderophore biosynthesis

As described above, the third siderophore biosynthetic step is performed by NRPSs. These enzymes have a modular structure where one module, the catalytic unit, is composed of an adenylation domain (A) for substrate specificity and activation, a peptidyl carrier (PCP) domain that binds a 4′phosphopantetheine cofactor for attachment of the activated substrate, and a condensation (C) domain for bond formation (Finking and Marahiel, 2004). As *Cryptococcus* species are not siderophore producers, NRPSs domains analysis was performed only with SidCp ortholog found in *P. brasiliensis* genomes. These analyses revealed that, as in *A. fumigatus*, the three domains essential for NRPS function are present in SidCp from the three *P. brasiliensis* isolates examined (**Figure 3A**). Domains found in other siderophore biosynthesis related proteins are shown in **Table A2** in Appendix.

Siderophore uptake

The presence of orthologs for appropriate siderophore genes and the fact that the iron binding capacity of medium from low iron cultures of *P. brasiliensis* is greater than that of iron-replete medium (Arango and Restrepo, 1988) supports our hypothesis that *P. brasiliensis* produces and captures siderophores from the extracellular environment. Therefore, we have categorized putative *P. brasiliensis* siderophore transporters by sequence homology analysis (**Table 2**; **Figure 1**). Searches of the *P. brasiliensis* genomes revealed that all three isolates contain the *S. cerevisiae* gene homolog SIT *sit1*. *S. cerevisiae* can utilize siderophore-bound iron either by the reductive iron-assimilation system or by membrane transporters. In the latter case, the uptake is mediated by four transporters that differ in substrate specificity: Sit1p/Arn3p, Arn1p, Taf1p/Arn2p, Enb1p/Arn4p (Lesuisse et al., 1998; Heymann et al., 1999, 2000; Yun et al., 2000a,b). Sit1p/Arn3p recognizes ferrioxamines, coprogen, and ferrichromes lacking anhydromevalonic acid. Additionally, *P. brasiliensis* isolates possess the *A. nidulans* SIT gene homologs, *mirB*, and *mirC* (**Table 2**; **Figure 1**). Heterologs expression assays of *A. nidulans mir* genes in a *S. cerevisiae* mutant strain unable to uptake siderophores have demonstrated that MirBp transports native TAFC, a hydroxamate siderophore. The growth of *P. brasiliensis* is stimulated by coprogen B and dimerum acid (DA), a derivative of rhodotorulic acid from *Blastomyces dermatitidis,* suggesting that *P. brasiliensis* can use hydroxamate compounds as iron sources (Castaneda et al., 1988).

The siderophore transporter Sit1p/Arn3p and the transporters of the SIT-family (*mirA*, *mirB* and *mirC*) were found in *C. neoformans* var. *grubii* and *C. gattii* (**Table 2**; **Figure 1**). The homolog gene *sit1/arn3* was previously identified in *C. neoformans* var. *neoformans* using SAGE employed to examine the transcriptome under iron-limiting and iron-replete conditions (Lian et al., 2005). Mutants defective in *sit1* had increased melanin production and elevated transcript levels for the laccase gene, *lac1*. The melanin phenotype may be caused by changes in iron homeostasis or membrane trafficking, perhaps leading to altered copper loading of laccase in the cell wall. Studies with mutants lacking *sit1/arn3* in *C. neoformans* var. *grubii* and *C. neoformans* var. *neoformans* have demonstrated that the gene *sit1* is required for siderophore utilization (ferrioxamine B) and growth in low iron-environments (Tangen et al., 2007). An overview of the siderophore biosynthesis and uptake in *P. brasiliensis* and *Cryptococcus* species is shown in **Figure 1**.

Analysis of transmembrane domains in siderophore–iron transporters

Amino acid sequences of siderophore transporter orthologs found in *P. brasiliensis* isolates and *Cryptococcus* species were analyzed in the TopPred server to predict their transmembrane domain topologies. **Figure 3B** presents the transmembrane segments of Sit1p in *S. cerevisiae*, *P. brasiliensis* isolates, *C. neoformans* var. *grubii* and *C. gattii*.

Table 2 | Orthologs to genes related to siderophore biosynthesis and to iron uptake by the non-reductive siderophore transport system in *P. brasiliensis* and *Cryptococcus* species.

Gene	Organism/ accession number	Predicted function	Orthologs in *Pb* 01, 03 and 18 (accession numbers)†	*E*-value*	Orthologs in *Cryptococcus* species (accession numbers)†	*E*-value*
sidA	*A. fumigatus*	Ornithine-N^5-	PAAG_01682.1	0.0	Not identified	–
	XP_755103	monooxygenase	PABG_03730.1	0.0		
			PADG_00097.1	0.0		
sidF	*A. fumigatus*	N^5-transacylases	PAAG_01680.1	0.0	Not identified	–
	XM_743567		PABG_03728.1	0.0		
			PADG_00100.1	0.0		
sidC	*A. fumigatus*	Non-ribosomal	PAAG_08527.1	0.0	Not identified	–
	XP_753088	peptide synthetase	PABG_04670.1	0.0		
			PADG_05295.1	0.0		
sidD	*A. fumigatus*	Non-ribosomal	PAAG_01679.1	0.0	CNAG_03588.2	e-40
	XP_748662	peptide synthetase	PABG_03726.1	0.0	CNBG_2041.2	e-41
			PADG_00102.1	0.0		
sidG	*A. fumigatus*	N^2-transacetylase	Not identified	–	CNAG_04355.2	2e-5
	XP_748685				CNBG_2703.2	e-6
sit1/arn3	*S. cerevisiae*	Siderophore	PAAG_06516.1	0.0	CNAG_00815.2	0.0
	NP_010849	transporter	PABG_02063.1	0.0	CNBG_1123.2	0.0
			PADG_00462.1	0.0		
mirA	*A. nidulans*	Siderophore	Not identified	–	CNAG_02083.2	0.0
	AY027565	transporter			CNBG_5232.2	0.0
mirB	*A. nidulans*	Siderophore	PAAG_01685.1	0.0	CNAG_07751.2	0.0
	XP_681809	transporter	PABG_03732.1	0.0	CNBG_2036.2	0.0
			PADG_00095.1	0.0		
mirC	*A. nidulans*	Siderophore	PAAG_02233.1	0.0	CNAG_07519.2	0.0
	AY135152	transporter	PABG_04747.1	0.0	CNBG_1087.2	e-44
			PADG_05373.1	0.0		

**Similarities with E-values < 10^{-5} were considered significant.*
†Accession numbers: PAAG refers to Pb01; PABG refers to Pb03; PADG refers to Pb18; CNAG refers to C. neoformans *var.* grubii *and CNBG refers to* C. gattii.

The number of segments varies between 12 and 15. Identical topology was found in Sit1p from *P. brasiliensis* isolates *Pb*03 and *Pb*18, whereas *Pb*01 has a different topology. Transmembrane domains were also identified in all the other siderophore transporters, as shown in **Table A2** in Appendix. These transporters also contain a MFS1 domain, which indicates that they belong to the MFS of transporters.

Iron source preferences

Several fungal pathogens utilize heme or hemoglobin as sources of iron (Foster, 2002; Jung et al., 2008). *C. albicans* expresses surface receptors for hemoglobin and hemolytic factors (Manns et al., 1994). Interestingly, heme–iron utilization in *C. albicans* is facilitated by Rbt5p, an extracellular glycosylphophatidylinositol (GPI)-anchored hemoglobin-binding protein (Weissman et al., 2008). Although there is no experimental evidence regarding the utilization of iron from the heme group by *P. brasiliensis*, there are genes that show similarity with Hmx-1p (Pendrak et al., 2004), and exhibit a heme oxygenase domain (PAAG_06626.1 in *Pb*01; PABG_02644.1 in *Pb*03; PADG_01082.1 in *Pb*18) in each of the *P. brasiliensis* isolates. These genes are annotated as conserved hypothetical or as predicted proteins. *C. neoformans* var. *grubii* is also able to utilize heme and hemoglobin as iron sources, but the mechanism(s) of heme utilization by this fungus are still unclear (Jung et al., 2008).

Transferrin has also been shown to be an iron source for both *C. albicans* and *C. neoformans* var. *grubii*. These fungi employ high-affinity permeases to acquire iron from transferrin in mammalian

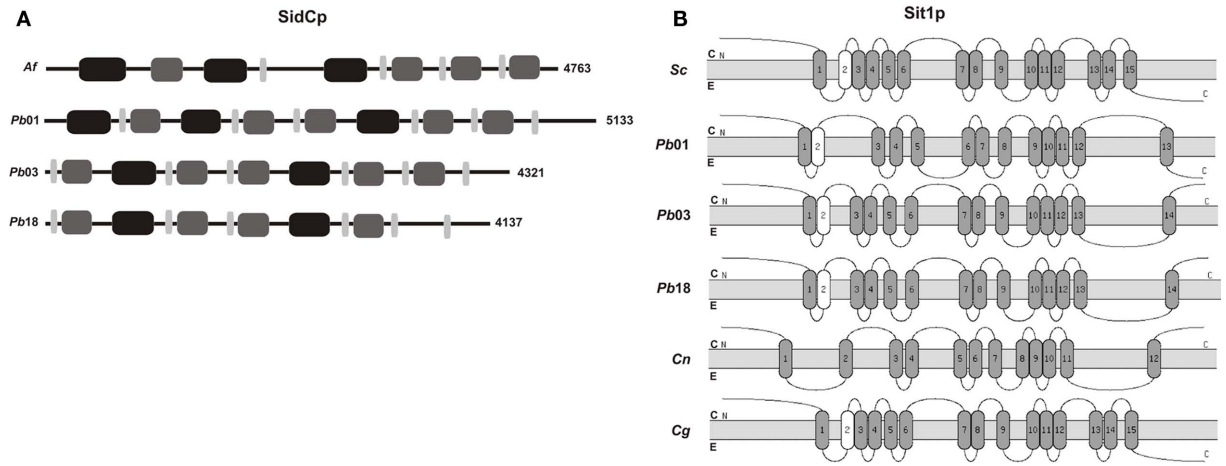

FIGURE 3 | Conserved features in proteins involved in biosynthesis and uptake of siderophores. (A) Modular organization of SidCp in *P. brasiliensis* isolates. Black boxes represent adenylation (A) domain, dark gray boxes illustrate the condensation (C) domain and light gray boxes represent the peptidyl carrier (PCP) domain. The length of each protein, in amino acids, is shown on the right. **(B)** Topology of transmembrane domains of Sit1p from *S. cerevisiae*, *P. brasiliensis* isolates and *Cryptococcus* species. White boxes represent putative segments, according to cutoff parameters (cutoff for certain transmembrane segments 1.00; cutoff for putative transmembrane segments 0.60). E: extracellular environment; C: cytosol. The topology prediction was performed using the TopPred server. Accession numbers in A: *Af* (XP_753088), *Pb*01 (PAAG_08527.1), *Pb*03 (PABG_04670.1), *Pb*18 (PADG_05295.1). Accession numbers in B: *Sc* (NP_010849), *Pb*01 (PAAG_06516.1), *Pb*03 (PABG_02063.1), *Pb*18 (PADG_00462.1), *Cn* (CNAG_00815.2), and *Cg* (CNBG_1123.2).

hosts through the reductive system (Knight et al., 2005; Jung et al., 2008). In the *P. brasiliensis* genome databases, genes were found (PAAG_04670.1; PABG_00038.1; PADG02428.1, respectively for isolates *Pb*01, *Pb*03 and *Pb*18) with high similarity to Cft1p, a permease from *C. neoformans* var. *grubii* required for iron utilization from transferrin (Jung et al., 2008).

COPPER

Copper uptake by the reductive system

Little is known about copper metabolism in *P. brasiliensis*. However, our *in silico* analyses of the *S. cerevisiae* copper metabolism-related genes in comparison to *P. brasiliensis* genomic databases revealed genes related to the copper reduction metalloreductase, *fre*. Copper transport is well described in *S. cerevisiae* where it is reduced from Cu (II) to Cu (I) by several cell surface metalloreductases encoded by several *fre* genes. These metalloreductases are regulated by iron and copper availability, mediated by the transcriptional factor Mac1p (Jungmann et al., 1993). Homologs of the copper metalloregulatory transcription factor gene (*mac*1) are present in both *Pb*01 and *Pb*03 genomes, but not in *Pb*18. Additionally, the high-affinity copper transport (Ctr3p) was found in all three isolate genomes. In *S. cerevisiae*, after reduction, copper is transported by the high-affinity copper transporter comprised by Ctr3p and Ctr1p, which are functionally redundant, although they have distinct amino acid sequences. Ctr3p is an integral membrane protein that assembles as a trimer to form a competent copper uptake permease at the plasma membrane. *S. cerevisiae* Ctr1p is localized at the plasma membrane and exists as an oligomer *in vivo*. These two high-affinity copper transport proteins are induced by copper deprivation and repressed by copper excess (Dancis et al., 1994a; Pena et al., 2000). In our *in silico* analyses, genes for the high-affinity copper transporter of the plasma membrane (*ctr1*) were not found, suggesting that high-affinity copper transport is performed only by the Ctr3p protein.

Genes related to metallochaperone (*atx*1), Cu^{2+} transporting P-type ATPase (*ccc2*) and superoxide dismutases (*sod1* and *sod2*; **Table 1**) were also found in *P. brasiliensis* genomes. In the cell, copper is transported by Atx1p, a cytosolic copper metallochaperone protein, that transports Cu (I) to Ccc2p, a transporting P-type ATPase containing a cytoplasmic region containing two distinct soluble metal-binding domains that interact with Atx1p (Banci et al., 2007). Ccc2p mediates the export of copper from the cytosol and distributes it to cupric proteins (Yuan et al., 1997). *S. cerevisiae* also has a detoxification pathway formed by Cup1p and Cup2p, metallothioneins (**Table 1**), that protect against copper poisoning (Hamer et al., 1985). An alternative copper transport system is mediated by Ctr2p, a vacuolar membrane protein of *S. cerevisiae*, that mobilizes vacuolar copper stores to cytosolic copper chaperones (Rees et al., 2004). Homologs of the low-affinity copper transporter of the vacuolar membrane (Ctr2p) are in *Pb*03 and *Pb*18, but not in *Pb*01. Additionally, the metallothioneins (encoded by *cup1* and *cup2* genes) were not identified in *P. brasiliensis* isolates *Pb*01, *Pb*03 and *Pb*18.

In silico analysis (**Table 1**) revealed that *Cryptococcus* species have orthologs encoding ferric/cupric reductases, suggesting that the copper reduction process is similar to that described for *S. cerevisiae*. Homologs of the high-affinity copper transporter *ctr3* gene and copper metalloregulatory transcription factor gene (*mac1*) have previously been identified (Waterman et al., 2007). Also, proteins with similarity to the cytosolic copper metallochaperone (*atx1* gene), the Cu^{2+} transporting P-type ATPase (*ccc2* gene) and the cytosolic and mitochondrial superoxide dismutases (*sod1* and *sod2* genes) have also identified, suggesting that copper distribution in *Cryptococcus* species occurs as described in *S. cerevisiae*. A homolog of the *ctr2* gene was identified only in *C. neoformans* var. *grubii*. Recently it was demonstrated that Ctr2p links copper homeostasis to polysaccharide capsule production in *C. neoformans*. The lack of this protein resulted in increased phagocytosis by murine macro-

phage, sensitivity to copper starvation and defects in polysaccharide capsule formation and melanization (Chun and Madhani, 2010). The gene *ctr1* for the high-affinity copper transporter of the plasma membrane and the genes *cup1* and *cup2* for metallothioneins were not found in *Cryptococcus* species. These analyses suggest that the high-affinity copper transport in cryptococcal cells is primarily performed by the protein encoded by *ctr3*.

Analysis of conserved motifs present in copper transporters

Searches for conserved domains revealed the presence of Mets and M*XXX*M motifs in the Ctr3p of the *P. brasiliensis* isolates and the *Cryptococcus* species (**Figure 4**). Studies in yeast and mammalian cells have revealed that proteins of the CTR family are integral membrane proteins containing three membrane-spanning domains, with high protein sequence homology (Dancis et al., 1994a; Lee et al., 2002). With the exception of *S. cerevisiae* Ctr3p, all CTR family members are rich in methionine residues within the amino-terminal portion (Labbe et al., 1999). These residues are arranged as M*XX*M and/or M*X*M, called Mets motifs, and it has been suggested that they could be involved in extracellular copper binding (Dancis et al., 1994b). It has been demonstrated that these clustered methionine residues together with an M*XXX*M motif in the transmembrane domain of CTR family members are important for copper uptake (Puig et al., 2002). In *P. brasiliensis* the M*XXX*M motif is found within the third transmembrane segment. The Ctr3p of *Cryptococcus* species contains only two predicted transmembrane domains instead of the three transmembrane segments described for other fungi. In *C. neoformans* var. *grubii* and *C. gattii*, the M*XXX*M motif is within the second transmembrane domain. Conserved domains were also found in amino acid sequences of other proteins involved in copper metabolism (**Table A1** in Appendix), suggesting that the orthologs found in *P. brasiliensis* and *Cryptococcus* may have activities that are similar to genes with established functions in other fungi.

ZINC

Zinc uptake

Comparisons to the *S. cerevisiae* genes related to zinc metabolism performed in *P. brasiliensis* genomes are presented in **Table 1**. Analyses demonstrate that *P. brasiliensis* has homologs to zinc transporters described in *S. cerevisiae* that are localized in the plasmatic, vacuolar and endoplasmic reticulum membranes. Importantly, five genes encoding to transporters of the ZIP family, with homology to *S. cerevisiae* Zrt1p or Zrt2p, are in the *P. brasiliensis* genomic database. In *S. cerevisiae,* zinc is transported by proteins belonging to the ZIP family, which is composed by a zinc high-affinity transporter protein encoded by the *zrt1* gene and a low-affinity transporter encoded by the *zrt2* gene (Gaither and Eide, 2001). We have previously identified homologs of zinc transporters by transcriptional analysis of *P. brasiliensis* yeast cells after incubation in human blood and plasma (Bailão et al., 2006, 2007). Interestingly, *P. brasiliensis* isolate *Pb*01 has two vacuolar membrane zinc transporters, encoded by the *zrc1* and *cot1* genes, whereas isolates *Pb*03 and *Pb*18 contain only the *cot1* homolog. Intracellularly, zinc is in vacuoles in association with the vacuolar membrane proteins Zrc1p and Cot1p, members of the cation diffusion facilitator (CDF) family (MacDiarmid et al., 2002). A homolog of the transcription factor Zap1p is also present in the three *P. brasiliensis* isolates. The expression of the genes associated with zinc homeostasis is positively regulated in *S. cerevisiae* by the transcription factor Zap1p, which regulates the expression of *zrt1*, *zrt2*, *zrt3*, *fet4*, and *zcr1* under zinc limiting conditions (Wu et al., 2008). Therefore, zinc assimilation in *P. brasiliensis* may be similar to that of *S. cerevisiae*.

Similarly, zinc homeostasis in *Cryptococcus* species is poorly studied. *In silico* analysis was performed by comparing *S. cerevisiae* genes related to zinc metabolism in genomic cryptococcal databases (**Table 1**). The results show that *C. neoformans* var. *grubii* and *C. gattii* have Zrt1p and Zrt2p zinc transporters homologs. These proteins putatively internalize zinc into the cell. Further, homologs of the vacuolar transporter Cot1p and the CDF Msc2p are present. Cot1p is presumably in the vacuolar membrane and should be related to zinc storage in this compartment. Msc2p, an endoplasmic reticulum membrane zinc transporter, could be related to zinc transport to this organelle. The protein encoded by *msc2* (CDF) is responsible for zinc homeostasis in the endoplasmic reticulum in *S. cerevisiae* (Ellis et al., 2004). A homolog of the transcription factor Zap1p is also present in *Cryptococcus*. Since homologs to the vacuolar membrane zinc transporter gene *zrt3* were not identified, the *zrc1* and *cot1* genes, encoding vacuolar membrane zinc transporters

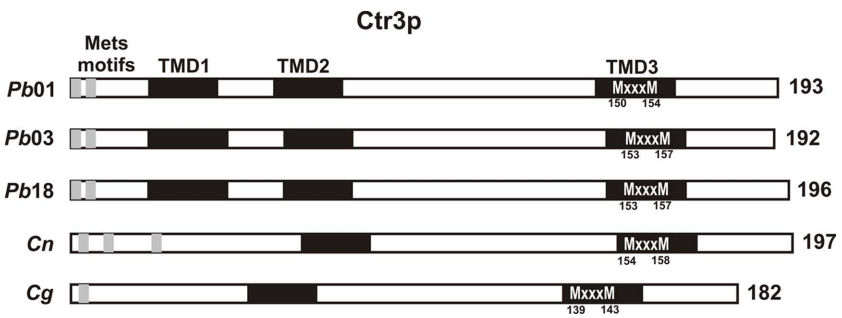

FIGURE 4 | Conserved features found in the primary structure of Ctr3p of *P. brasiliensis* isolates and *Cryptococcus* species. Ctr3p from *P. brasiliensis* isolates contains three putative transmembrane domains (TMD1-3, shown in black) while Ctr3p from *Cryptococcus* species presents only two TMDs. All species contain putative copper binding motifs (Mets motifs) arranged as M*XX*M and/or M*X*M. M*XXX*M motif in TMD3 in *P. brasiliensis* isolates and TMD2 in *Cryptococcus* species are represented in white characters. The length of each protein, in amino acids, is shown on the right. Accession numbers: *Pb*01 (PAAG_05251.1), *Pb*03 (PABG_07607.1), *Pb*18 (PADG_05084.1), *Cn* (CNAG_00979.2) and *Cg* (CNBG_0560.2).

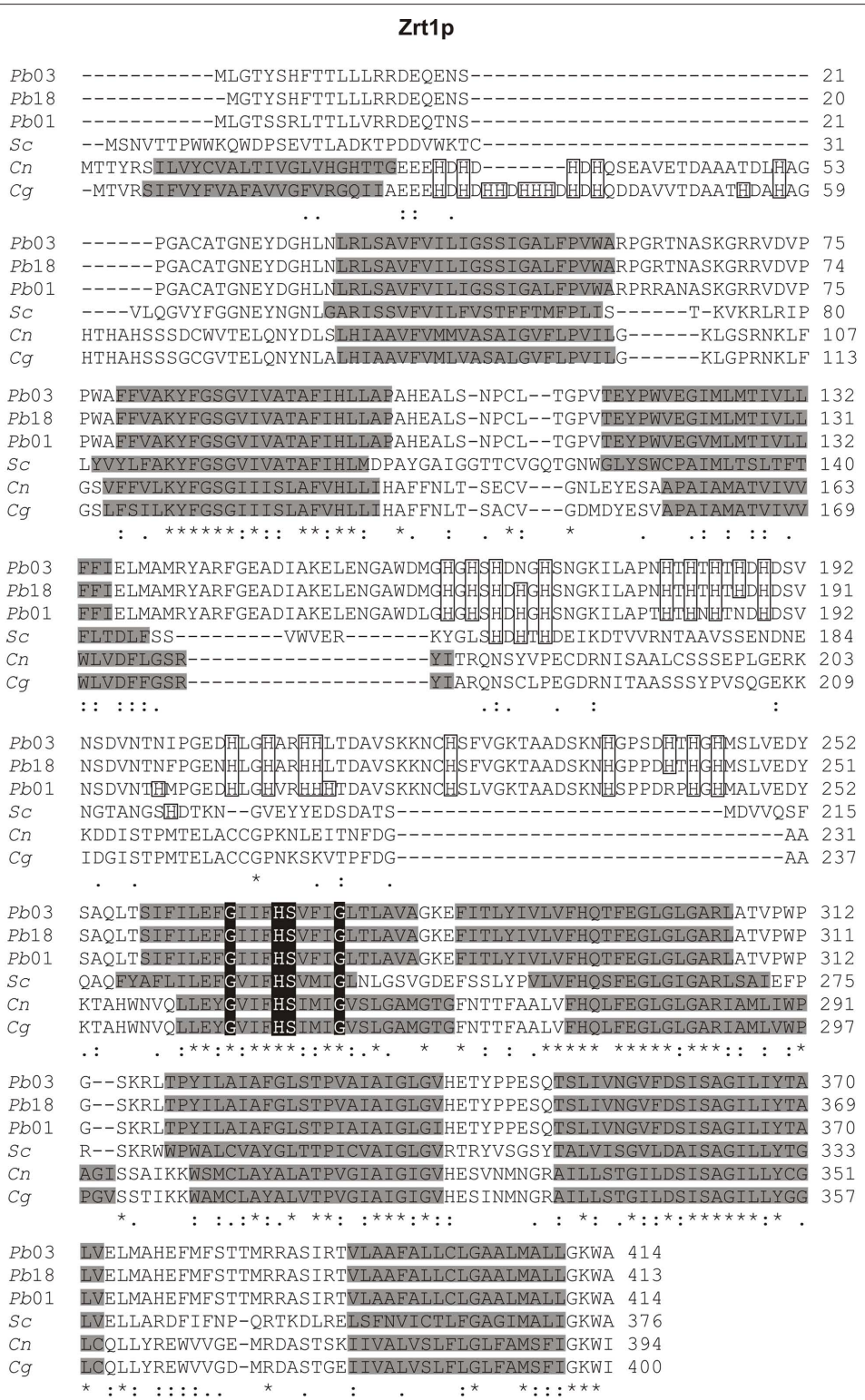

FIGURE 5 | Alignment of amino acid sequences of Zrt1p from *S. cerevisiae*, *P. brasiliensis* isolates and *Cryptococcus* species. The predicted transmembrane domains are shown in gray boxes. The black boxes inside the transmembrane segment contain conserved histidine-serine and glycine residues. The histidines found in the amino-terminal region of Zrt1p from *Cryptococcus* species and in the loop between transmembrane domains III and IV in *P. brasiliensis* and *S. cerevisiae* are boxed. Asterisks indicate amino acid identity and dots represent conserved substitutions. Accession numbers: *Pb*03 (PABG_07725.1), *Pb*18 (PADG_08567.1), *Pb*01 (PAAG_08727.1), *Sc* (NP_011259), *Cn* (CNAG_03398.2) and *Cg* (CNBG_2209.2).

could be responsible for the zinc transport to this organelle. This analysis suggests that *C. neoformans* var. *grubii* and *C. gattii* could obtain zinc via routes similar to that described for *S. cerevisiae*.

Analysis of conserved regions in the high-affinity zinc transporter (Zrt1p) in P. brasiliensis *isolates and* Cryptococcus *species*

Alignment of Zrt1p amino acid sequence from *S. cerevisiae*, *P. brasiliensis* isolates and *Cryptococcus* species revealed some conserved features (**Figure 5**). Concerning the predicted transmembrane domain number, all *P. brasiliensis* isolates contain eight predicted domains, while both *C. neoformans* var. *grubii* and *C. gattii* have nine. Proteins belonging to the ZIP family are predicted to have from five to eight transmembrane domains and they vary in size from 233 to 477 amino acid residues. The variations in the amino-terminal portion are usually responsible for the differences in size. The transmembrane domain IV has the most conserved portions of ZIP family proteins, with conserved histidine and glycine residues. The histidine residue and the adjacent polar residue, usually a serine, within the transmembrane domain are predicted to comprise part of a heavy metal-binding site in the center of the membrane (Eng et al., 1998). The amino acid sequence of *S. cerevisiae* Zrt1p presents a number of histidine residues in a large loop between the transmembrane segments III and IV, which is a putative metal ion binding site (Zhao and Eide, 1996a). The histidine-serine and glycine residues are conserved within the fourth transmembrane region in *P. brasiliensis* and within the fifth transmembrane region in *Cryptococcus*. Regarding the histidine rich region, it is conserved between transmembrane domains III and IV in *P. brasiliensis* isolates, whereas are conserved at the amino-terminal portion in *Cryptococcus* species, as occurs in other members of the ZIP family (Eng et al., 1998). Conserved domains are also found in amino acid sequences of other proteins involved in zinc metabolism that were identified in the search for orthologs (**Table A1** in Appendix).

CONCLUSION

As we have described, microorganisms are extremely well equipped to exploit host metal sources during growth and infection. *Cryptococcus* species demonstrate remarkable flexibility in gaining access to and utilizing iron, the most investigated micronutrient in this organism. Our laboratories have begun to elucidate the mechanisms for the uptake and metabolism of micronutrients such as iron, copper and zinc in *P. brasiliensis*. Studies on individual genes and pathways are revealing unique features of micronutrients metabolism in this fungus. The application of systems biology approaches that incorporates genomic and proteomic data will further generate hypotheses about the common and specific responses to micronutrient deprivation in both pathogenic fungi and potentially lead to the development of novel therapeutics exploiting their metal requirements.

ACKNOWLEDGMENT

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APPENDIX

Table A1 | Conserved domains in proteins involved in iron, copper and zinc uptake by reductive systems in *P. brasiliensis* isolates and *Cryptococcus* species.

Gene product	Predicted function	Organism/accession number†	Conserved domains*	Transmembrane domains*	Signal peptide*
Fre1	Metalloreductase	*P. brasiliensis* 01/PAAG_05370.1	Ferric reductase domain	7	Yes
		P. brasiliensis 03/PABG_06003.1	FAD-binding domain	6	No
			NAD-binding domain		
Fre3	Metalloreductase	*P. brasiliensis* 01/PAAG_02079.1	Ferric reductase domain	6	Yes
		P. brasiliensis 03/PABG_02329.1	FAD-binding domain	6	Yes
		P. brasiliensis 18/PADG_00813.1	NAD-binding domain	6	Yes
Fre5	Metalloreductase	*P. brasiliensis* 03/PABG_07812.1	Ferric reductase domain	6	No
			FAD-binding domain		
			NAD-binding domain		
Fre7	Metalloreductase	*P. brasiliensis* 01/PAAG_06164.1		8	No
		P. brasiliensis 03/PABG_06497.1	Ferric reductase domain	8	No
		P. brasiliensis 18/PADG_07957.1	FAD-binding domain	8	No
		C. neoformans/CNAG_00876.2	NAD-binding domain	7	No
		C. gattii/CNBG_6082.2		8	No
Fre8	Metalloreductase	*C. neoformans*/CNAG_07334.2	Ferric reductase domain	6	No
			FAD-binding domain		
		C. gattii/CNBG_2116.2	NAD-binding domain	6	No
Fre10	Metalloreductase	*C. neoformans*/CNAG_06821.2	Ferric reductase domain	4	No
			FAD-binding domain		
		C. gattii/CNBG_5888.2	NAD-binding domain	4	No
Cfl4	Metalloreductase	*C. neoformans*/CNAG_06524.2	Ferric reductase domain	5	No
			FAD-binding domain		
			NAD-binding domain		
Frp1	Metalloreductase	*P. brasiliensis* 01/PAAG_04493.1	Ferric reductase domain	5	No
		P. brasiliensis 03/PABG_04278.1	FAD-binding domain	6	No
		P. brasiliensis 18/PADG_04652.1	NAD-binding domain	5	No
Fet3	Ferroxidase	*C. neoformans* CNAG_06241.2	Copper-oxidase domain	1	Yes
Fet5	Ferroxidase	*P. brasiliensis* 03/PABG_05667.1	Copper-oxidase domain	–	No
		P. brasiliensis 18/PADG_05994.1		–	No
		C. neoformans/CNAG_07865.2		1	Yes
		C. gattii/CNBG_4942.2		1	Yes
Fet31	Ferroxidase	*P. brasiliensis* 01/PAAG_06004.1	Copper-oxidase domain	1	No
		C. neoformans/CNAG_02958.2		–	Yes
Fet33	Ferroxidase	*P. brasiliensis* 01/PAAG_00163.1	Copper-oxidase domain	–	No
		P. brasiliensis 03/PABG_05183.1		–	Yes
Ftr1/Ftr2	Iron permease	*C. neoformans*/CNAG_06242.2	FTR1 domain	7	Yes
		C. gattii/CNBG_3602.2		6	Yes
Fth1	Iron permease	*C. neoformans*/CNAG_02959.2	FTR1 domain	7	Yes
		C. gattii/CNBG_4943.2		7	Yes

(Continued)

Table A1 | Continued

Gene product	Predicted function	Organism/accession number†	Conserved domains*	Transmembrane domains*	Signal peptide*
Smf1	Low-affinity Permease	*C. neoformans*/CNAG_05640.2	Nramp domain	11	No
		C. gattii/CNBG_6162.2		11	No
Ccc1	Vacuolar transporter	*P. brasiliensis* 01/PAAG_07762.1	DUF125 domain	4	No
		P. brasiliensis 03/PABG_00362.1		4	No
		P. brasiliensis 18/PADG_02775.1		4	No
		C. neoformans/CNAG_05154.2		4	No
		C. gattii/CNBG_4540.2		4	No
Mrs3/Mrs4	Mitochondrial iron transporter	*P. brasiliensis* 01/PAAG_05053.1	Mitochodrial carrier domain	–	No
		P. brasiliensis 03/PABG_04509.1		–	No
		P. brasiliensis 18/PADG_04903.1		–	No
		C. neoformans/CNAG_02522.2		–	No
		C. gattii/CNBG_4218.2		–	No
Yfh1	Mitochondrial matrix iron chaperone	*P. brasiliensis* 01/PAAG_02608.1	Frataxin domain	–	No
		P. brasiliensis 03/PABG_03095.1		–	No
		P. brasiliensis 18/PADG_01626.1		–	No
		C. neoformans/CNAG_05011.2		–	No
		C. gattii/CNBG_4670.2		–	No
Ggt1	Secreted glutathione-dependent ferric reductase	*P. brasiliensis* 01/PAAG_06130.1	Gamma-glutamyltranspeptidase domain	1	Yes
		P. brasiliensis 03/PABG_06527.1		1	Yes
		P. brasiliensis 18/PADG_07986.1		1	Yes
		C. neoformans/CNAG_02888.2		–	No
		C. gattii/CNBG_3537.2		–	No
Mac1	Copper metalloregulatory transcription factor	*P. brasiliensis* 01/PAAG_08210.1	Copper fist domain	–	No
		P. brasiliensis 03/PABG_07429.1		–	No
		C. neoformans/CNAG_07724.2		–	No
		C. gattii/CNBG_2252.2		–	No
Ctr3	High-affinity copper transporter of the plasma membrane	*P. brasiliensis* 01/PAAG_05251.1	Ctr domain	3	No
		P. brasiliensis 03/PABG_07607.1		3	No
		P. brasiliensis 18/PADG_05084.1		3	No
		C. neoformans/CNAG_00979.2		2	No
		C. gattii/CNBG_0560.2		2	No
Ctr2	Putative low-affinity copper transporter of the vacuolar membrane	*P. brasiliensis* 03/PABG_01536.1	Ctr domain	3	No
		P. brasiliensis 18/PADG_04146.1		3	No
		C. neoformans/CNAG_01872.2		3	No
Atx1	Cytosolic copper metallochaperone	*P. brasiliensis* 01/PAAG_00326.1	HMA domain	–	No
		P. brasiliensis 03/PABG_06615.1		–	No
		P. brasiliensis 18/PADG_02352.1		–	No
		C. neoformans/CNAG_02434.2		–	No
		C. gattii/CNBG_4136.2		–	No
Ccc2	Cu^{2+} transporting P-type ATPase	*P. brasiliensis* 01/PAAG_07053.1		7	No
		P. brasiliensis 03/PABG_03057.1	HMA domain	8	No

(Continued)

Table A1 | Continued

Gene product	Predicted function	Organism/accession number†	Conserved domains*	Transmembrane domains*	Signal peptide*
		P. brasiliensis 18/PADG_01582.1	Hydrolase domain	8	No
		C. neoformans/CNAG_06415.2	E1-E2 ATPase domain	8	No
		C. gattii/CNBG_5045.2		8	No
Sod1	Cytosolic superoxide dismutase	*P. brasiliensis* 01/PAAG_04164.1	SOD domain	–	No
		P. brasiliensis 03/PABG_03954.1		–	No
		P. brasiliensis 18/PADG_07418.1		–	No
		C. neoformans/CNAG_01019.2		–	No
		C. gattii/CNBG_0599.2		–	No
Sod2	Mitochondrial superoxide dismutase	*P. brasiliensis* 01/PAAG_02725.1	SOD N-terminal domain	–	No
		P. brasiliensis 03/PABG_03204.1	SOD C-terminal domain	–	No
		P. brasiliensis 18/PADG_01755.1		–	No
		C. neoformans/CNAG_04388.2		–	No
		C. gattii/CNBG_2661.2		–	No
Zrt1	High-affinity zinc transporter of the plasma membrane	*P. brasiliensis* 01/PAAG_08727.1	Zip domain	8	No
		P. brasiliensis 03/PABG_07725.1		8	No
		P. brasiliensis 18/PADG_08567.1		8	No
		C. neoformans/CNAG_03398.2		9	Yes
		C. gattii/CNBG_2209.2		9	Yes
Zrt2	Low-affinity zinc transporter of the plasma membrane	*P. brasiliensis* 01/PAAG_03419.1	Zip domain	8	Yes
		P. brasiliensis 03/PABG_05498.1		7	No
		P. brasiliensis 18/PADG_06417.1		8	Yes
		C. neoformans/CNAG_00895.2		8	Yes
Zrc1	Vacuolar membrane zinc transporter	*P. brasiliensis* 01/PAAG_00702.1	Cation efflux domain	6	Yes
Cot1	Vacuolar membrane zinc transporter	*P. brasiliensis* 01/PAAG_07885.1	Cation efflux domain	5	Yes
		P. brasiliensis 03/PABG_07467.1		4	No
		P. brasiliensis 18/PADG_08196.1		5	Yes
		C. neoformans/CNAG_02806.2		6	Yes
		C. gattii/CNBG_3460.2		4	Yes
Zrt3	Vacuolar membrane zinc transporter	*P. brasiliensis* 01/PAAG_09074.1	Zip domain	6	No
		P. brasiliensis 03/PABG_04697.1		6	No
		P. brasiliensis 18/PADG_05322.1		6	No
Msc2	Cation diffusion facilitator protein of the endoplasmic reticulum and nucleus	*P. brasiliensis* 03/PABG_07115.1	Cation efflux domain	10	No
		P. brasiliensis 18/PADG_06381.1		10	No
		C. neoformans/CNAG_05394.2		11	No
		C. gattii/CNBG_4458.2		10	No
Zap1	Zinc-regulated transcription factor	*P. brasiliensis* 01/PAAG_03645.1	Zinc finger C_2H_2 domain	–	No
		P. brasiliensis 03/PABG_03305.1		–	No
		P. brasiliensis 18/PADG_01870.1		–	No
		C. neoformans/CNAG_05392.2		–	No
		C. gattii/CNBG_4460.2		–	No

**Amino acid sequence analysis was performed using the online software SMART.*

†Accession numbers: PAAG refers to Pb01; PABG refers to Pb03; PADG refers to Pb18; CNAG refers to C. neoformans *var.* grubii *and CNBG refers to* C. gattii.

Table A2 | Conserved domains in proteins related to siderophore biosynthesis and to iron uptake by the non-reductive siderophore transport system in *P. brasiliensis* isolates and *Cryptococcus* species.

Gene product	Predicted function	Organism/accession number†	Conserved domains*	Transmembrane domains*	Signal peptide*
SidA	Ornithine-N^5-monooxygenase	*P. brasiliensis* 01/PAAG_01682.1	Pyr_redox_2 domain	–	No
		P. brasiliensis 03/PABG_03730.1		–	No
		P. brasiliensis 18/PADG_00097.1		–	No
SidF	N^5-transacylases	*P. brasiliensis* 01/PAAG_01680.1	AlcB domain	–	No
		P. brasiliensis 03/PABG_03728.1		–	No
		P. brasiliensis 18/PADG_00100.1		–	No
SidC	Non-ribosomal peptide synthetase	*P. brasiliensis* 01/PAAG_08527.1	Adenylation domain	–	No
		P. brasiliensis 03/PABG_04670.1	Peptidyl carrier domain	–	No
		P. brasiliensis 18/PADG_05295.1	Condensation domain	–	No
SidD	Non-ribosomal peptide synthetase	*P. brasiliensis* 01/PAAG_01679.1	Adenylation domain	–	Yes
		P. brasiliensis 03/PABG_03726.1	Peptidyl carrier domain	–	No
		P. brasiliensis 18/PADG_00102.1		–	No
		C. neoformans/CNAG_03588.2	Condensation domain	–	No
		C. gattii/CNBG_2041.2		–	No
SidG	N^2-transacetylase	*C. neoformans*/CNAG_04355.2	MYND-type zinc finger domains	–	No
		C. gattii/CNBG_2703.2	Acetyltransferase domain	–	No
Sit1/Arn3	Siderophore transporter	*P. brasiliensis* 01/PAAG_06516.1	MFS1 domain	12	No
		P. brasiliensis 03/PABG_02063.1		14	No
		P. brasiliensis 18/PADG_00462.1		14	No
		C. neoformans/CNAG_00815.2		13	No
		C. gattii/CNBG_1123.2		13	No
MirA	Siderophore transporter	*C. neoformans*/CNAG_02083.2	MFS1 domain	12	No
		C. gattii/CNBG_5232.2		11	No
MirB	Siderophore transporter	*P. brasiliensis* 01/PAAG_01685.1	MFS1 domain	14	No
		P. brasiliensis 03/PABG_03732.1		14	No
		P. brasiliensis 18/PADG_00095.1		14	No
		C. neoformans/CNAG_07751.2		14	No
		C. gattii/CNBG_2036.2		14	No
MirC	Siderophore transporter	*P. brasiliensis* 01/PAAG_02233.1	MFS1 domain	8	No
		P. brasiliensis 03/PABG_04747.1		12	No
		P. brasiliensis 18/PADG_05373.1		12	No
		C. neoformans/CNAG_07519.2		10	No
		C. gattii/CNBG_1087.2		14	Yes

**Amino acid sequence analysis was performed using the online software SMART.*

†Accession numbers: PAAG refers to Pb01; PABG refers to Pb03; PADG refers to Pb18; CNAG refers to C. neoformans *var.* grubii *and CNBG refers to* C. gattii.

III. ARTIGO PUBLICADO NA REVISTA CURRENT FUNGAL INFECTION REPORTS

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GENOMICS AND PATHOGENESIS (S SHOHAM, SECTION EDITOR)

Metal Acquisition and Homeostasis in Fungi

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Abstract Transition metals, particularly iron, zinc and copper, have multiple biological roles and are essential elements in biological processes. Among other micronutrients, these metals are frequently available to cells in only limited amounts, thus organisms have evolved highly regulated mechanisms to cope and to compete with their scarcity. The homeostasis of such metals within the animal hosts requires the integration of multiple signals producing depleted environments that restrict the growth of microorganisms, acting as a barrier to infection. As the hosts sequester the necessary transition metals from invading pathogens, some, as is the case of fungi, have evolved elaborate mechanisms to allow their survival and development to establish infection. Metalloregulatory factors allow fungal cells to sense and to adapt to the scarce metal availability in the environment, such as in host tissues. Here we review recent advances in the identification and function of molecules that drive the acquisition and homeostasis of iron, copper and zinc in pathogenic fungi.

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Introduction

Metals such as iron, copper and zinc have numerous biological roles and play a central role at the host–pathogen interface. Mammalian and microbial cells have an essential demand for these metals, which act as both structural and catalytic cofactors for proteins, and are therefore required for biological processes. During infection, the competing demands for these nutrients culminate in a struggle for metal acquisition/utilization at the microbe–host interface [1•, 2•]. In the complex interactions between pathogens and their mammalian hosts, metal homeostasis plays an essential role in both virulence and host defense [3, 4].

Iron and copper participate in several oxidation–reduction reactions because of their ability to lose and gain electrons. This same property permits iron and copper to generate reactive oxygen species (ROS) [5, 6]. Zinc is also an essential cofactor of many enzymes, but in excess, may be toxic to cells [7]. For metal balance, cells usually regulate uptake, storage and consumption. Our understanding of the mechanisms involved in metal excretion is incomplete. This review summarizes the current knowledge regarding the most studied metals that contribute to virulence of fungal pathogens: iron, copper and zinc. We focus on the fungal pathogens *Candida albicans*, *Histoplasma capsulatum*, *Aspergillus fumigatus*, *Cryptococcus neoformans* and *Paracoccidioides*. Specifically we discuss the struggle for control of transition metals during infection, the molecular mechanisms involved in iron, copper and zinc uptake and the regulation of metal homeostasis in those pathogens. Additionally we review the preferential host iron sources

and fungal genes related to iron acquisition/homeostasis directly involved in infection.

Host Metal Homeostasis During Infectious Processes

Among metals involved in fungal infection, the functions of iron are well characterized. Hosts have evolved mechanisms to efficiently acquire iron and at the same time decrease its availability to pathogens [2•]. Physiological conditions that lead to metal overload contribute to increased infections. For example, administration of exogenous iron results in exacerbation of cryptococcosis [8] and increases in free iron also results in higher fungal load in mouse tissues infected with *Paracoccidioides* [9]. At the interface between iron and immunity, macrophages appear as a cellular factory that manage metal homeostasis [3]. Upon infection, the iron efflux from macrophages is suppressed resulting in 70 % reduction in plasma iron, thus restricting the amount of the metal available to extracellular pathogens. Infected macrophages, conversely, restrict the amount of iron available to intracellular microbes by pumping out iron via the ferroportin transporter route. Mutation-impaired ferroportin function compromises the ability of macrophages to clear pathogens [10].

Lactoferrin is produced by neutrophils and epithelial cells to chelate iron in extracellular compartments resulting in impairment of proliferation of fungal invaders [11]. Induction of ferritin production to facilitate withholding of intracellular iron diminishes the amount of the metal available to intracellular pathogens [2•, 12]. Iron also influences immune functions mediated by macrophages, and cytokines affect systemic iron homeostasis and cellular iron efflux [13]. Reduced iron levels have been found in macrophages activated by exposure to interferon gamma (IFN-γ) or granulocyte macrophage colony-stimulating factor (GM-CSF) [14]. Transferrin can be used by pathogens as an iron source in host tissues. To counteract this process IFN-γ decreases the expression of transferrin receptor in macrophages. Moreover, the production of the cellular iron storage molecule ferritin can be regulated by proinflammatory signals [15]. So, in the complex host–pathogen interaction, the control of iron homeostasis is a battlefield where the host must withdraw the micronutrient from microbes and at the same time uses iron to elaborate an efficient oxidative burst, since this metal is required for generation of ROS.

Since copper is essential, it is not unexpected that both humans and pathogens share the requirement for acquiring sufficient levels of copper [6]. In response to fungal infection, macrophages phagocytose the fungal cells and initiate cellular events that culminate in the oxidative burst [6]. Studies suggest that fungal pathogens must obtain copper to develop an efficient survival mechanism in host tissues, since genes related to copper acquisition/homeostasis are upregulated during infection [16, 17]. *C. neoformans* fights the host defenses to acquire copper, which promotes melanin synthesis, a virulence factor for this fungus [18]. The dependence of fungi upon copper for survival under the host conditions can be related to their response to ROS generation by the host since superoxide dismutase is a copper-dependent enzyme.

Zinc levels are modulated during infectious processes. During inflammation, the liver sequesters zinc, likely limiting zinc bioavailability to pathogenic microbes [19]. Neutrophils display an antimicrobial mechanism based on competition for zinc. This zinc-chelating system, found in neutrophil cytoplasm and abscess fluid, exerts fungistatic activity based on the calcium- and zinc-binding protein calprotectin [20]. Abscess fluid inhibits the growth of several fungi and the addition of zinc results in fungal growth in this fluid [7], reinforcing the view that zinc sequestration is a strategy used by the host to combat fungal infections. A metallomic study has demonstrated that GM-CSF-activated macrophages reduce intracellular zinc concentrations upon *H. capsulatum* infection in order to kill the pathogen [14].

Molecular Mechanisms of Iron, Copper and Zinc Uptake

Iron uptake mechanisms are highly regulated in fungi since excess iron is toxic and iron excretion systems have not yet been described in fungi [21]. Fungi have evolved different mechanisms for iron acquisition [21]. A low-affinity iron uptake system characterized only in *Saccharomyces cerevisiae* involves permeases that transport not only iron, but also other metals. In the reductive high-affinity ferrous uptake, ferrireductases reduce ferric iron (Fe^{3+}) to its soluble ferrous form (Fe^{2+}). Fe^{2+} is then reoxidized by plasma membrane ferroxidases and Fe^{3+} is promptly internalized by a high-affinity permease [5]. Another high-affinity mechanism for iron uptake is mediated by siderophores, small molecules with high affinity for Fe^{3+}, that allow specific recognition and uptake of iron at the cell surface [22]. Most fungi produce and secrete hydroxamate-type siderophores under low-iron growth conditions [23]. Some fungi, such as *C. neoformans*, do not produce siderophores, but can transport molecules produced by other organisms (xenosiderophores) [24].

The *C. albicans* genome contains genes that encode 18 putative ferrireductases and five ferroxidase homologues [1•, 25]. The ferroxidase Fet34 localizes to the plasma membrane and possibly associates with the permease Ftr1 early in the secretory pathway, promoting the high-affinity iron uptake [26•]. *C. albicans* produces a siderophore transporter [27] that displays broad substrate specificity, transporting various hydroxamate-type siderophores [28].

Under iron-limiting conditions, *H. capsulatum* produces three different reductants: a secreted glutathione dependent γ-glutamyltransferase (Ggt1) [29], non-enzymatic reductants with low molecular weight, and cell surface ferric reducing agents [30]. The *H. capsulatum* genome contains genes that encode seven putative ferrireductases [31]. Although a high-affinity acquisition mechanism has not been described for *H. capsulatum*, genomic analysis of the strain G186AR revealed genes coding iron permease (ftr1) and ferroxidase (Fet3) homologues [32]. *H. capsulatum* is also able to produce multiple hydroxamate siderophores under conditions of low iron availability [33]. In addition, *H. capsulatum* can utilize xenosiderophores [34].

In *A. fumigatus*, the ferrireductase FreB has been characterized. After reduction, iron is internalized by the ferroxidase-permease complex FetC–FtrA [35]. *A. fumigatus* synthesizes three types of siderophores, two of which are responsible for iron storage [36–38]. The iron-loaded siderophore is internalized by specific transporters [39] and the ester bonds of triacetylfusarinine C are then hydrolyzed by an esterase [40]. The cleavage products (fusarinines) are excreted, and the free iron can either be used in cell metabolism or bind to intracellular siderophore desferri-ferricrocin for storage [37, 38, 41•].

Uptake of iron is probably mediated by two large groups of transporters in *C. neoformans*: high- and low-affinity systems [42]. Cft1 is a high-affinity iron permease associated with the reductive system. On the other hand *cft2* possibly encodes for a low-affinity uptake system, since no clear iron-related phenotypes could be detected in *cft2* null mutants [43]. Cfo1 and Cfo2 ferroxidases have also been described in *C. neoformans* [44]. Cfo1 is required for high-affinity and reductive iron transport, since mutants lacking the coding gene show reduced growth under low iron conditions and cannot use ferric iron for growth. Moreover, under low iron conditions, Cfo1 expression is increased and localized mainly in the cell surface [44]. Studies have shown the inability of *Cryptococcus* species to produce siderophores. This is supported by genomic analysis, which has revealed the absence of genes involved in steps of siderophore biosynthesis [24, 45]. Despite the inability to synthesize siderophores, *Cryptococcus* species are presumably able to transport xenosiderophores [43].

Molecular mechanisms for reductive iron uptake in the genus *Paracoccidioides* are coming to light. In silico analysis has revealed that the genome of this fungus contains genes that encode redundant ferrireductase homologues [45]. Experiments have demonstrated a significant increase in the expression of genes coding the ferrireductases *fre3*, *fre7*, *frp1* and *ggt1* upon iron restriction (Fig. 1a). *Paracoccidioides* has glutathione-dependent ferrireductase activity [46], an aspect that is corroborated by the presence of a *ggt1* homologue in the fungus genome [45]. Since iron permease homologues were not detected in the *Paracoccidioides* genome, it has been proposed that a zinc permease could function additionally as an iron permease to acquire this metal [45]. The importance of iron acquisition by siderophores in the *Paracoccidioides* genus have been noted and studies on siderophore production and uptake are in

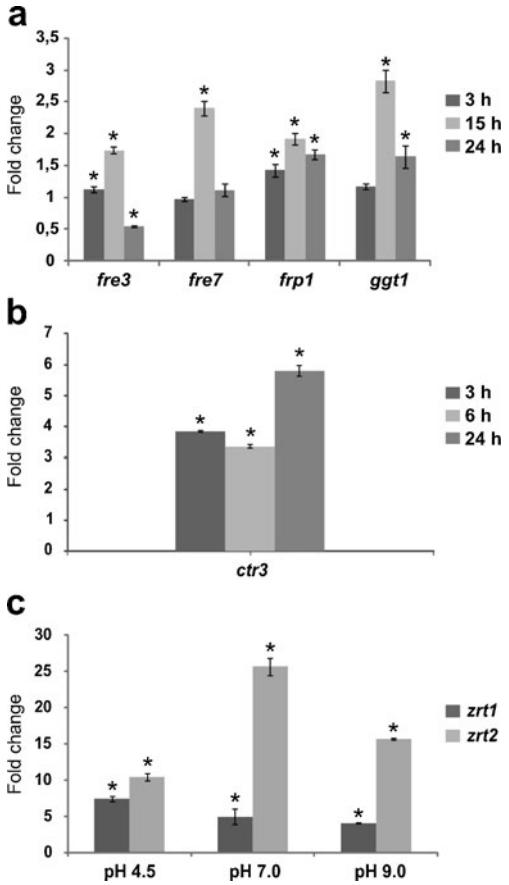

Fig. 1 Expression profile of *Paracoccidioides* (*Pb*01) genes during iron, copper and zinc starvation. *Pb*01 yeast cells were incubated in chemically defined medium containing different concentrations of iron, copper or zinc. Cells were harvested and total RNA was extracted using Trizol and mechanical cell rupture. After in vitro reverse transcription, the cDNAs were submitted to quantitative RT-PCR. The expression values were calculated using the transcripts alpha tubulin or l34 as endogenous controls [9, 82]. Data are presented as fold change relative to experimental controls. **a** Expression of ferrireductases encoding transcripts *fre3*, *fre7*, *frp1*, and *ggt1* evaluated in yeast cells in medium containing 3.5 μM iron (experimental control) or no iron for 3 h, 15 h and 24 h. **b** Expression of copper transporter encoding transcript *ctr3* evaluated in yeast cells in medium containing 50 μM copper (experimental control) or under conditions of copper starvation produced by adding the copper chelator bathocuproine disulphonate (BCS) for 3 h, 6 h and 24 h. **c** Expression level of zinc transporters encoding transcripts *zrt1* and *zrt2* evaluated in yeast cells in medium containing 30 μM zinc (experimental control) or under conditions of zinc depletion produced by adding the zinc chelator *N*,*N*,*N*,*N*-tetrakis(2-pyridylmethyl)ethylenediamine (TPEN) at different pH values (4.5, 7.0 and 9.0) for 6 h. Data are presented as means ± SD from triplicate determinations. *$p \leq 0.05$, *t* test, in relation to the data obtained from the experimental controls

progress. The major evidence of iron capture by siderophores is supported by the stimulation of fungal growth in the presence of coprogen B and dimerum acid [47]. In silico analysis ha revealed the presence of genes putatively involved in hydroxamate-type siderophore biosynthesis and transport [45]. Corroborating these data, it has been demonstrated by chrome azurol S assays that *Paracoccidioides* is a hydroxamate producer (Silva et al., personal communication).

In fungi, copper and iron homeostasis must be intrinsically linked since iron uptake requires ferroxidases, which are members of the multicopper oxidase family. Copper is first reduced by plasma membrane ferrireductases and then Cu^{1+} is internalized via a high-affinity permease [48]. In *C. albicans*, the ferrireductase c*fl1*/*fre1* is transcriptionally regulated in response to both iron and copper availability [49], indicating that this ferrireductase is also important in copper uptake. Furthermore, the mutant for the *ctr1* copper transporter displays deficient growth in medium low in copper and iron indicating that in *C. albicans* iron and copper homeostasis are linked [50].

As observed for iron, copper plays fundamental roles in several aspects of *C. neoformans* biology. For instance, the production of melanin pigment is dependent on a copper oxidase [51] and the copper-containing ferroxidases are necessary for iron uptake [44]. Copper is probably reduced in *C. neoformans* by the same enzymes that reduce iron at the cell surface [52]. Two copper transporters have been described in *C. neoformans*. The *ctr1* null mutant shows reduced growth in copper-depleted medium. Ctr4, by contrast, is not essential for cryptococcal development in low-copper medium. However, mutant cells lacking both *ctr1* and *ctr4* transporters display severe growth defects in copper-deprived environments [53•].

The *Paracoccidioides* genome contains genes that encode redundant ferrireductases [45] as cited above, suggesting that these enzymes could function as iron and copper reductases. Furthermore a high-affinity copper transporter, *ctr3*, is present at increased levels during copper shortage (Fig. 1b), reinforcing the view that Ctr3 could be involved in copper uptake in *Paracoccidioides* [45].

The zinc uptake system in most fungi comprises just high-affinity and low-affinity permeases belonging to the ZIP family [54, 55], since this metal does not need to be reduced before internalization. Eight genes encoding proteins of the ZIP family of zinc transporters have been described in *A. fumigatus* [56]. Expression of *zrfA*, *zrfB* and *zrfC* is regulated by both zinc and pH [56, 57]. ZrfA and ZrfB function under acidic, zinc-limiting conditions. It seems that ZrfB is a high-affinity zinc permease, since a *zrfB* transcript was downregulated under high zinc conditions [58]. ZrfC participates in zinc uptake in a neutral or alkaline, zinc-poor environment [56]. Aspf2 putatively contributes to zinc uptake as a zinc-binding protein localized in the fungal periplasm [56].

Paracoccidioides possesses two zinc permease homologues (*zrt1* and *zrt2*), indicating a specific zinc uptake system [45]. The transcriptional response of *zrt* homologues to zinc starvation has been demonstrated by quantitative RT-PCR (Fig. 1c). The *zrt2* transcript, but not *zrt1* transcript, is highly expressed at neutral to alkaline pH during zinc depletion (Fig. 1c), as observed to *A. fumigatus* ZrfC.

Host Iron Sources

A high proportion of circulating iron in humans exists as heme in hemoglobin and hemin, iron-containing porphyrins. *C. albicans* shows hemolytic activity, and membrane proteins capable of binding hemin/hemoglobin have been identified [1•]. *C. albicans* Rbt5, a glycosylphosphatidylinositol-anchored protein, is the major hemoglobin receptor [59]. *hmx1* encodes an intracellular heme oxygenase that breaks down iron-protoporphyrin IX to α-biliverdin and is required for heme-iron utilization [60]. In silico analysis has revealed that *Paracoccidioides* genome contains genes that encode *hmx1* and *rbt5* homologues, suggesting effective hemoglobin iron acquisition by this fungus [45].

Intracellular iron in humans is bound to ferritin. *C. albicans* hyphae are able to obtain iron from ferritin using Als3 protein as a receptor. Als3 is a multifunctional protein since it can also function as an adhesin and an invasin [1•]. Transferrin is a glycoprotein that transports iron in serum. *C. albicans* is able to take up iron from transferrin by the reductive pathway using the ferrous permease Ftr1 and ferrireductase Fre10 [61]. In *H. capsulatum*, ferrireductase activity is higher in the presence of hemin and transferrin, suggesting that this fungus uses the ferrireductases to obtain iron during infection [34]. *Paracoccidioides* is likely to be able to take up iron from transferrin since the fungus has five genes encoding ferrireductases in the genome [45].

Regulation of Iron, Copper and Zinc Homeostasis in Pathogenic Fungi

Fungi have evolved sophisticated control mechanisms for maintenance of optimal levels of iron, copper and zinc. These mechanisms include the regulation of genes involved in metal ion uptake, utilization and storage. In fungi, metal ion homeostasis is mainly achieved by transcriptional regulation of gene expression. A group of iron-responsive GATA-type transcription factors mediates repression of iron acquisition genes in response to iron

sufficiency [62]. These regulators have a cysteine-rich central domain located between two zinc fingers, which directly interact with iron [63].

A range of genes and regulators involved in the response of *C. albicans* to iron starvation have been described [64]. During iron sufficiency, the GATA-type regulator Sfu1 downregulates expression of *arn1* and *hap43* genes encoding a siderophore transporter and a transcription factor, respectively [64, 65]. Under iron-limited conditions, the Cap2 protein represses the expression of Sfu1, activating genes of iron uptake pathways [66•]. Sef1 and Rim101 were also identified as positive regulators of iron acquisition in *C. albicans* [67, 68].

A GATA-type factor, Sre1, has been described in *H. capsulatum*. Sre1 acts as a negative regulator of siderophore biosynthesis genes in response to iron excess [69]. Sre1 also regulates cellular processes other than iron acquisition, such as optimal filamentous growth [70]. The same occurs with the Sre1 homologue SreB in *Blastomyces dermatitidis*. SreB regulates siderophore biosynthesis and also governs phase transition and cell growth at 22 °C in *B. dermatitidis* [71].

During iron sufficiency, high-affinity iron uptake systems (reductive pathway and siderophore production) are repressed by SreA in *A. fumigatus* [72]. During iron starvation, the *A. fumigatus* bZIP-type regulator HapX represses iron-dependent pathways, such as respiration, TCA cycle and heme biosynthesis, to save iron, and activates iron uptake by siderophores [73•]. Thus the transcription factors SreA and HapX act in opposite ways within the cell depending on the environmental iron status. During iron excess, SreA is activated and represses HapX expression, while during iron paucity, HapX represses the expression of SreA. In *A. fumigatus* the transcription factor AcuM stimulates iron acquisition via HapX induction and SreA repression [74].

C. neoformans Cir1 possesses a cysteine-rich domain, but unlike other fungal GATA-type iron regulators, it has only a zinc finger motif [75]. Cir1 is a global transcription factor which senses iron levels and regulates positively and negatively the transcriptional response [75, 76]. The expression of *C. neoformans* virulence attributes, such as capsule formation, growth at host temperature and melanin production, are also controlled by Cir1 [75]. A post-translational mechanism for the control of the amount of Cir1 suggests that under conditions of iron starvation Cir1 protein levels decrease. In contrast, iron availability promotes Cir1 stabilization and consequent repression of iron acquisition genes [77]. The transcriptional response to iron in *C. neoformans* is also regulated by HapX. As well as Cir1, HapX has both a positive and negative influence in the regulation of gene expression. However, unlike Cir1, HapX plays a modest role during infection and probably is important during environmental iron acquisition [78].

Proteomic analysis has revealed that during iron starvation the metabolic status of the pathogenic fungus *Paracoccidioides* is altered. Glycolysis is upregulated while iron-consuming pathways, such as tricarboxylic and glyoxylate cycles, are repressed. It has been demonstrated that under iron-limited conditions the transcript level of the HapX increases [9]. However, the regulatory mechanisms that orchestrate the global changes in response to iron availability in this fungus have not been described and are the subject of current investigation.

Regulatory mechanisms that respond to copper availability among pathogenic fungi have been best studied in *C. neoformans*. The copper-dependent transcription factor, Cuf1, has a cysteine-rich sequence, which contains a putative copper binding motif [79]. Under conditions of copper limitation, Cuf1 induces the expression of the copper transporter encoding genes *ctr1* and *ctr4*. During copper excess, the metallothionein (copper binding and detoxifying protein) genes *cmt1* and *cmt2* are induced by Cuf1 [53•]. A copper-dependent transcriptional regulator, Mac1, found in *C. albicans*, is transcriptionally autoregulated and activates the expression of *ctr1* and *fre7* genes during copper paucity [80].

Although regulation of copper homeostasis has not yet been described in *Paracoccidioides*, studies have revealed that the high-affinity copper transporter, Ctr3, is upregulated under infection conditions [16, 81] and is also a potential adhesin [82]. Analysis of genes potentially involved in copper regulation has demonstrated the presence of a copper metalloregulatory transcription factor, Mac1, in *Paracoccidioides* [45], thus prompting further investigation.

As for copper, the regulation of zinc homeostasis in pathogenic fungi is poorly understood. A zinc-responsive transcription factor has been described in *C. albicans* [83]. The Zap1/Csr1 factor induces expression of the plasma membrane zinc transporters, Zrt1 and Zrt2, and is also involved in the control of efficient hyphae and biofilm matrix formation and production of quorum sensing molecules [83–86].

In *A. fumigatus* the expression of *zrfA* and *zrfB* is induced by the ZafA zinc-responsive transcriptional activator under zinc-limited conditions [87]. However, under neutral zinc-limited conditions, the expression of these transporters is repressed by the transcriptional regulator PacC [57]. Additionally, the expression of *zrfC* is upregulated by ZafA under zinc-limited conditions regardless of the environmental pH and downregulated by PacC under acidic growth conditions [56].

Although zinc metabolism regulation is not well understood in *Paracoccidioides* and *Cryptococcus* pathogens, a homologue of Zap1 zinc-regulated transcription factor has been found in their genomes [45]. Studies focusing on this potential transcriptional regulator are in progress.

Virulence

Despite the close correlation between metal availability and virulence, the fungal genes related to iron acquisition/homeostasis directly involved in host infection are poorly described [88, 89]. Table 1 lists metal acquisition/homeostasis genes and provides information on their role in virulence.

In *C. albicans,* mutants lacking the iron permease coding gene *ftr1* lose virulence [90]. The involvement of genes related to siderophore uptake in virulence was not observed [27, 28]. Moreover virulence attenuation was observed in *C. albicans* mutants lacking the iron-responsive transcriptional regulators *hap43*, *aft2*, *sef1* and *cap2* and the heme oxygenase coding gene *hmx1* [65, 66•, 91, 92, 93•], indicating that all these genes are important during *C. albicans* infection.

Table 1 Roles of genes involved in metal homeostasis and virulence of pathogenic fungi

Gene	Protein function	Role in virulence	Reference
Candida albicans			
ftr1	High-affinity iron permease	The *ftr1Δ* mutatation results in complete loss of the capacity to damage epithelial cells in vitro. Moreover mutants lacking *ftr1* are avirulent in mice infected with *C. albicans* during the early stationary phase	[90]
hap43	Transcriptional regulator	Deletion of *hap43* attenuates the virulence of *C. albicans* in a mouse model of disseminated infection	[65]
cap2	Transcriptional regulator	The *cap2Δ* mutant shows delayed virulence in a mouse model of *C. albicans* infection	[66•]
aft2	Transcriptional regulator	The *aft2Δ*/*aft2Δ* strain shows attenuated virulence in mice with disseminated infection	[91]
hmx1	Heme oxygenase	The homozygous mutant *hmx1Δ*/*hmx1Δ* shows reduced virulence in mice with disseminated infection	[93•]
sef1	Transcriptional regulator	The *sef1Δ* mutant shows significantly decreased virulence compared to wild-type strain in BALB/c mice with disseminated infection	[92]
Aspergillus			
sidA	Involved in siderophore biosynthesis	The *sidAΔ* mutant shows completely attenuated virulence in mice	[37, 94]
sidD	Involved in siderophore biosynthesis	The *sidDΔ* mutant shows severely attenuated virulence in neutropenic mice	[95]
sidF	Involved in siderophore biosynthesis	The *sidFΔ* mutant shows attenuated virulence in neutropenic mice infected intranasally	[95]
hapX	Transcriptional regulator	The *hapXΔ* mutant shows attenuated virulence in immunosuppressed mice	[73•]
acuM	Transcriptional regulator	The *acuMΔ* mutant shows attenuated virulence in neutropenic mice with disseminated infection and invasive pulmonary aspergillosis, resulting in significantly delayed mortality	[74]
zafA	Transcriptional regulator	The *zafAΔ* mutant shows reduced virulence in immunosuppressed mice infected intranasally	[87]
pacC	Transcriptional regulator	The *Aspergillus nidulans pacCΔ* mutant shows attenuated virulence in immunosuppressed mice	[99]
Histoplasma capsulatum			
sid1	Involved in siderophore biosynthesis	The *sid1Δ* strain shows a significant defect in pulmonary colonization compared to wild-type cells in mice infected intranasally	[31]
Cryptococcus neoformans			
cft1	High-affinity iron permease	The *cft1Δ* mutant shows attenuated virulence and reduced fungal burden	[43]
cfo1	Ferroxidase	The *cfo1Δ* mutant shows significantly attenuated virulence in mice	[44]
cir1	Transcriptional regulator	The *cir1Δ* mutant is avirulent in mice	[75]
ctr4	Copper transporter	The *ctr4Δ* null mutant shows reduced spread to tissues and is completely avirulent in infected mice	[79]
ctr1	Copper transporter	*ctr1Δ* mutant presented reduced melanization, reduced capsule and enhanced phagocytosis index	[97]
clc	Chloride channel	The *clc-A* mutant shows attenuated virulence in a mouse cryptococcosis model, since *clc-A* plays a role in capsule and laccase expression, important virulence factors	[98]
ccc2	Copper transporter	*ccc2* mutation results in absence of melanization, an important virulence factor	[18]
cuf1	Transcriptional regulator	The deletion of *cuf1* results in attenuated virulence in a mouse model of cryptococcosis	[79]

In *Aspergillus*, mutants lacking genes involved in siderophore biosynthesis (*sid*) have pointed to the relevance of this iron uptake pathway to virulence. *sidA* deletion in *A. fumigatus* abolishes siderophore biosynthesis and completely attenuates virulence [37, 94]. A similar effect was observed in mutants lacking the *sidA* homologue, *sid1*, in *H. capsulatum* [31]. Genes related to fusarinine C and triacetylfusarinine C production, *sidD* and *sidF,* significantly affect *A. fumigatus* virulence [95]. Furthermore, the deletion of the transcriptional regulators *hapX* and *acuM* causes significant attenuation of virulence in a murine model of infection [73•, 74].

The role of iron acquisition in *Cryptococcus* virulence has been extensively studied in recent years. Almost all *C. neoformans* genes involved in iron homeostasis that have been analyzed are related to cryptococcal virulence, as evaluated in murine models of cryptococcosis using null gene mutants [44, 75–78]. When considering the iron permeases Cft1 and Cft2, virulence attenuation and reduced fungal burden are observed in the *cft1* gene null mutant and in the *cft1*/*cft2* double mutant, but not in the *cft2* knockout strain [76]. The ferroxidase Cfo1 also plays a role in virulence, since null mutants are also attenuated in virulence [44]. In addition, *C. neoformans cir1* null mutants are completely avirulent in murine models of cryptococcosis, which is consistent with the hypocapsular phenotype and its reduced ability to proliferate at 37 °C [75].

Regarding copper, a pivotal biological role of this metal has been already described for *C. neoformans*, since two proteins involved in virulence, Cu/Zn-Sod1 and laccase, require copper as a cofactor for activity [51, 96]. The *ctr4* gene is expressed during infection and is directly associated with virulence, since null mutants show reduced spread to tissues and are completely avirulent in infected mice [79]. The *ctr1* gene also is associated with virulence, as mutants show reduced melanization, a reduced capsule, and an enhanced phagocytosis index [97]. Moreover, *C. neoformans* strains with mutation in genes encoding copper distribution transporters, such as the *clc* chloride channel and the *ccc2* secretory transporter, show reduced virulence or reduced expression of virulence factors [18, 98]. Furthermore, in a mouse model of cryptococcosis, transcriptional regulator *cuf1* null mutants display disruption of several virulence-linked characteristics, such as reduced laccase activity, severe growth defects in low-copper medium, and reduced virulence [79]. Cuf1 is required for infection of the brain but not of the lung in mouse models of cryptococcosis, suggesting that copper is limiting in neurological infections [79].

Studies investigating the role of zinc during pathogenesis are sparse. Investigations are restricted to the importance of zinc-responsive transcription factors during pathogenesis, such as *zaf*A. In *A. fumigatus,* ZafA regulates zinc homeostasis, and mutants lacking this gene show reduced virulence in mice [87]. A similar result was found for the pH-responsive transcriptional factor *pac*C, that plays an essential role in pulmonary infection by *A. fumigatus* [56, 99].

Conclusions

Iron, copper and zinc acquisition is a critical determinant in fungal pathogenesis. To circumvent metal sequestration by the host during infection, pathogenic fungi have evolved mechanisms of metal acquisition. Understanding of the roles of iron, copper and zinc in fungal pathogenicity has advanced in recent years. As discussed above, fungi demonstrate remarkable flexibility in gaining access to and utilizing the transition metals iron, copper and zinc. The sophisticated acquisition and regulation of homeostasis of these metals are surely an efficient weapon facilitating fungal survival within the human host, and represent an important component of virulence.

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IV. RESULTADOS AINDA NÃO PUBLICADOS

Análises do genoma revelaram que *Paracoccidioides* spp. possui vários homólogos de redutases férricas (SILVA et al., 2011). Para avaliar se as enzimas codificadas por esses genes identificados *in silico* são funcionais em *Paracoccidioides* spp., foi realizado um ensaio qualitativo em fase sólida com a linhagem *Pb*01. O fungo apresentou atividade extracelular de redutase férrica, a qual se mostrou visivelmente maior na presença do quelante de ferro ácido etilenodiamino tetra-acético (EDTA) **(Figura 1)**, indicando que *Paracoccidioides* sp. pode possuir uma via redutiva ativa.

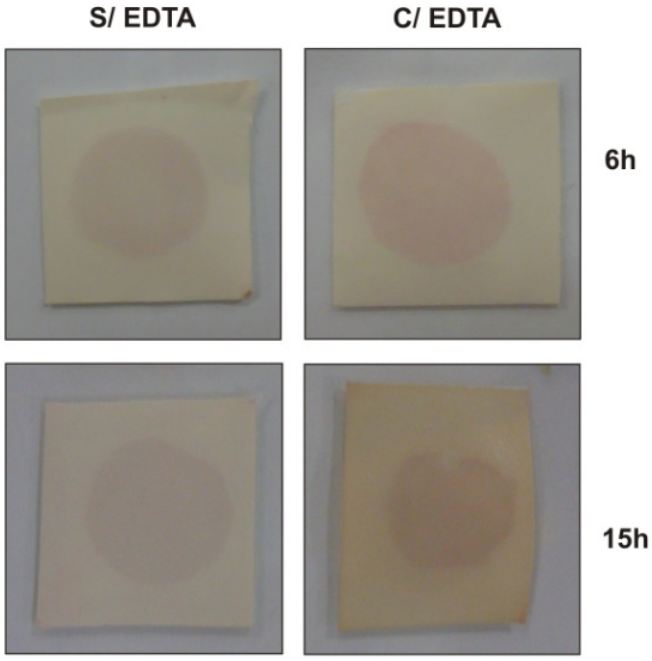

Figura 1. Atividade de redutase férrica de células leveduriformes de *Paracoccidioides* sp. Colônias de células leveduriformes de *Pb*01 foram crescidas sobre membranas de náilon e estas foram dispostas na superfície de meio sólido MMcM suplementado com Fe^{3+} na presença ou na ausência de EDTA, usado como quelante de ferro. Após 6 ou 15 h de incubação a 36 ^{o}C, as membranas foram retiradas e reveladas com um tampão contendo ácido dissulfônico da batofenantrolina (BPS). As membranas ficam coradas de vermelho quando há a formação de um complexo BPS-Fe^{2+}, indicando que houve atividade de redutase férrica extracelular, que converteu Fe^{3+} presente no meio de cultura em Fe^{2+} que se ligou ao BPS.

Para avaliar possíveis estratégias que *Paracoccidioides* sp. utiliza para manter a homeostase de ferro durante a privação do metal, foram realizadas análises quantitativas da expressão de genes possivelmente envolvidos com esses mecanismos, por RT-PCR em tempo real. Os dados obtidos do

fungo cultivado em meio MMcM sem adição do metal foram comparados aos dados obtidos do fungo cultivado em meio MMcM com adição de 3,5 μM de ferro [meio mínimo padrão, segundo Restrepo e Jimenez (1980)]. Os genes transportadores de zinco e ferro, Zrt1 e Zrt2 (SILVA et al., 2011), são induzidos em 15 h e 24 h, respectivamente, de privação de ferro **(Figura 2, painel A)**. Entre as ferroxidases, o gene *lacIV*, tem um aumento de expressão em 24 h na ausência do metal **(Figura 2, painel B)**, assim como *zrt2*. Os dados sugerem que a expressão dos genes analisados é regulada por ferro.

Com relação aos mecanismos de homeostase intracelular de ferro, foi observada uma diminuição significativa da expressão do gene *ccc1*, envolvido com a captação do metal para dentro do vacúolo, a partir de 3 h na ausência do metal **(Figura 2, painel C)**. O gene *mrs3/4* apresentou um aumento de expressão a partir de 15 h na ausência do metal, enquanto a expressão de *yfh1* não parece ser fortemente regulada nas condições testadas em *Paracoccidioides* sp. **(Figura 2, painel C)**. Esses dados sugerem que a expressão dos genes *ccc1* e *mrs3/4* é também regulada por ferro.

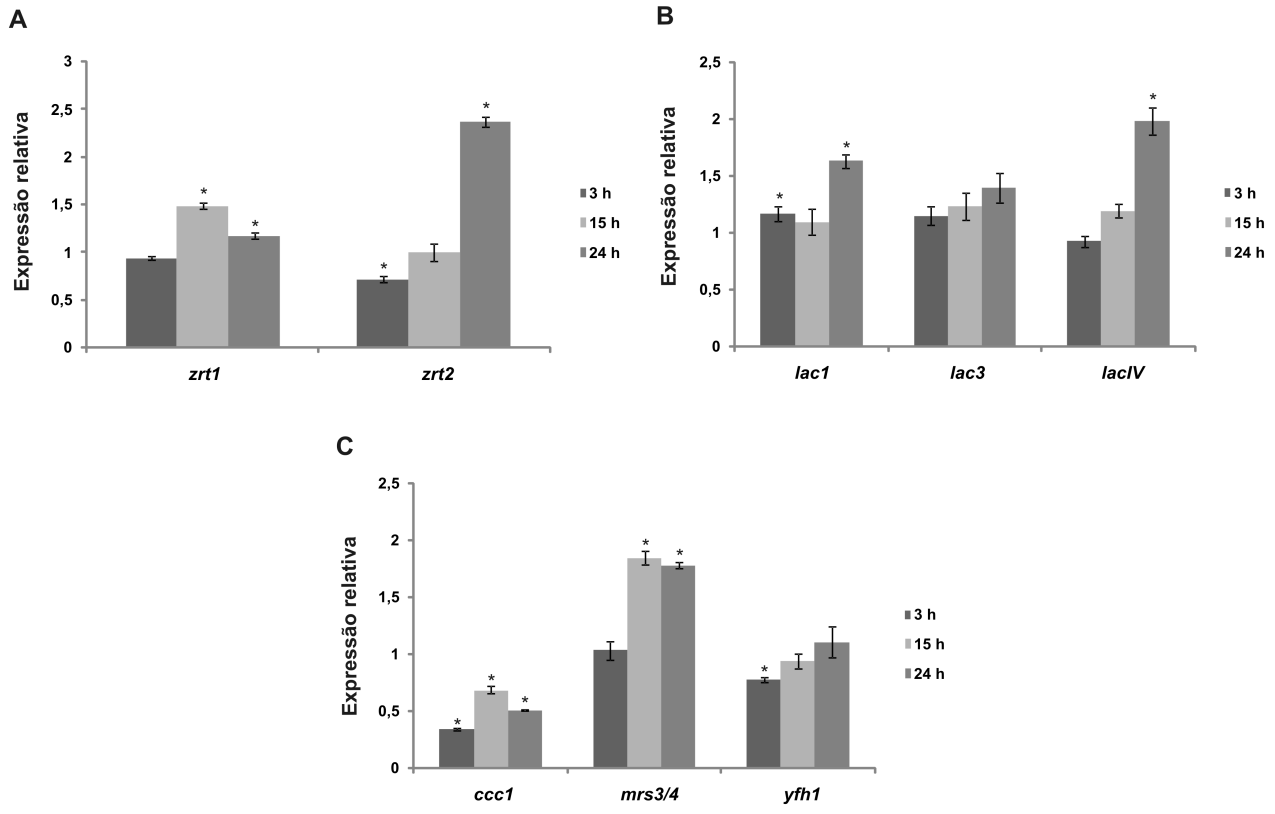

Figura 2. Cinética de expressão dos genes possivelmente envolvidos com a captação de ferro pela via redutiva e com a homeostase intracelular desse metal em *Paracoccidioides* sp. Células leveduriformes de

*Pb*01 foram incubadas em MMcM sem adição de ferro (teste) ou com 3,5 μM de ferro (controle experimental) por 3, 15 e 24 h. O RNA total das células submetidas a essas diferentes condições de cultivo foi obtido. Após as síntese dos cDNAs, foram avaliados os níveis de expressão de genes possivelmente codificantes para transportadores de ferro e zinco **(painel A)**, ferroxidases **(painel B)**, transportador de Fe^{2+}/Mn^{2+} vacuolar (*ccc1*), transportador de ferro da membrana mitocondrial interna (*mrs3/4*) e regulador da homeostase mitocondrial de ferro (*yfh1*) **(painel C)**. Os valores da expressão dos genes para a condição teste foram normalizados contra a condição controle (expressão relativa). A expressão dos genes analisados foi normalizada contra a expressão do gene *α-tubulina* (controle endógeno) nas mesmas condições. Os dados representados no gráfico expressam a média ± o desvio padrão das triplicatas. *Amostras com aumento ou diminuição estatisticamente significativa ($p < 0,05$) com relação ao seu respectivo controle experimental, pelo teste t de Student.

Para avaliar a taxa de captação de ferro por *Paracoccidioides* spp., ensaios com ferro radioativo (^{59}Fe) foram realizados. Para isso, células leveduriformes das linhagens *Pb*01 e *Pb*18 foram incubadas em meio SC suplementado ou não com 200 μM do quelante de ferro ácido dissulfônico da batofenantrolina (BPS), ou com 10 μM de $FeCl_3$. Essas células foram coletadas independentemente e submetidas ao ensaio de captação de ^{59}Fe na presença ou não do agente redutor ácido ascórbico. Na presença deste composto, avalia-se a taxa de transporte de Fe^{2+}, uma vez que o metal já encontra-se em sua forma reduzida. Já na ausência de ácido ascórbico, avalia-se a taxa de captação de ferro, o que requer tanto redutases férricas quanto transportadores (EIDE et al., 1992).

Foi observado que células, tanto de *Pb*01 quanto de *Pb*18, cultivadas na presença de BPS apresentaram uma taxa de captação de Fe^{2+} superior à taxa de captação do fungo cultivado nas outras condições. Em *Pb*18, a captação de Fe^{2+} apresentou-se claramente ferro-dependente em relação às condições de cultivo do fungo. As células cultivadas em meio suplementado com 10 μM de ferro apresentaram uma taxa de captação de Fe^{2+} menor do que a taxa observada para as células cultivadas em meio SC sem suplementação de ferro (~ 0,08 μM de ferro), e menor ainda do que a taxa observada para células cultivadas na presença de 200 μM de BPS **(Figura 3)**.

Quando ácido ascórbico não foi adicionado e a taxa de captação de ferro dependente de redutase férrica foi avaliada, apenas leveduras de *Pb*18 cultivadas na presença de BPS apresentaram captação de ferro **(Figura 3)**. Esses dados sugerem que o sistema de redutases férricas, apesar de existente em *Pb*01, pode ser pouco eficiente nesta linhagem. Já para *Pb*18, os dados sugerem que o

sistema de redutase férrica acoplado a um transportador de ferro só está ativo em condições limitantes do metal.

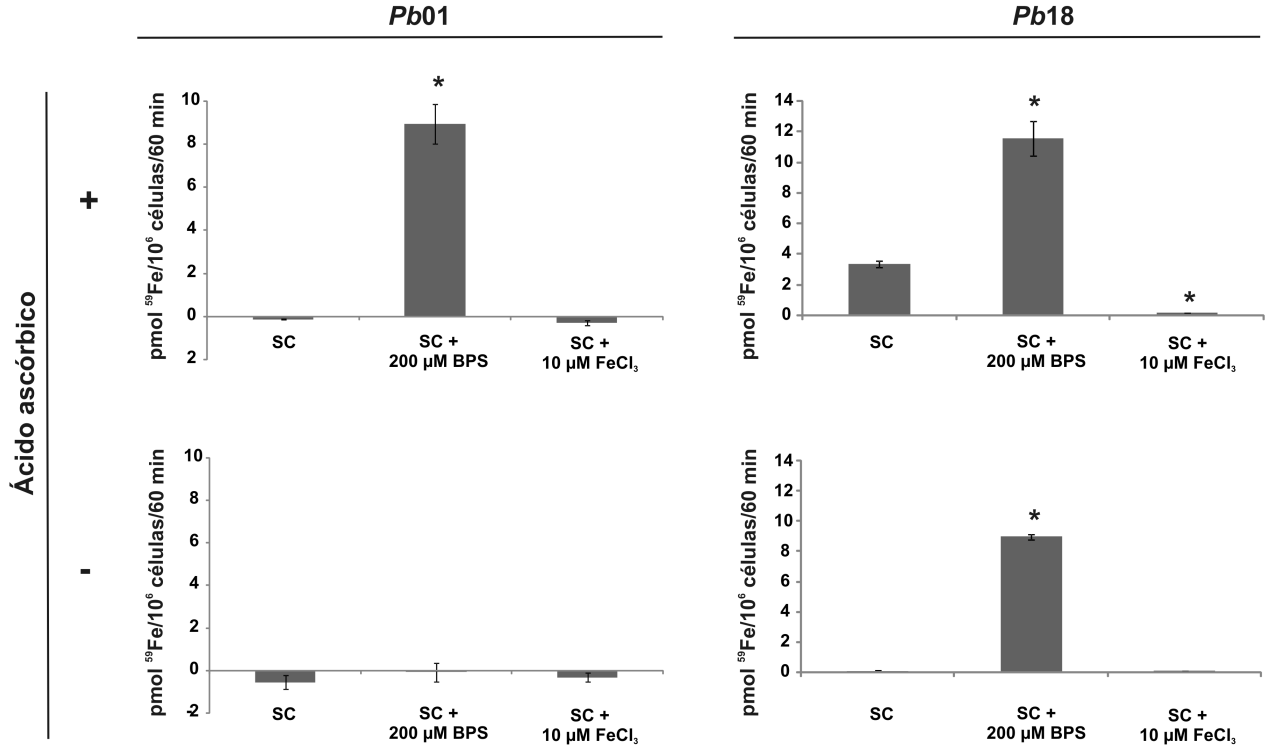

Figura 3. A taxa de captação de ferro por *Paracoccidioides* spp. é dependente das condições de cultivo do fungo e da forma do metal disponível no meio. As linhagens *Pb*01 e *Pb*18 foram cultivadas em meio SC suplementado ou não com 200 µM de BPS ou com 10 µM de $FeCl_3$ por 24 h. Após esse período, as células foram coletadas, lavadas e incubadas na presença de ^{59}Fe,com adição (+) ou não (-) de ácido ascórbico, por 60 min. Posteriormente, as células foram novamente coletadas e lavadas, e submetidas a um contador de radiação γ para determinação da velocidade de captação de^{59}Fe. Os dados estão apresentados como a média ± o desvio padrão das triplicatas ou quadruplicatas. *Amostras com aumento ou diminuição estatisticamente significativa ($p < 0,05$) com relação às células cultivadas em meio SC, pelo teste t de Student.

Como as células cultivadas na presença de BPS foram as que apresentaram maior taxa de captação de ferro, essa condição foi escolhida para dar continuidade a este estudo. Já foi reportado que compostos contendo Pt^{2+} inibem a atividade de algumas redutases férricas, mas não a captação de Fe^{2+} (já em sua forma reduzida) (EIDE et al., 1992). Para testar o efeito da adição de um composto contendo platina (PtII) sobre a captação de ferro por *Paracoccidioides* spp., células das linhagens *Pb*01 e *Pb*18 cultivadas na presença de 200 µM de BPS foram submetidas a diferentes

condições de captação de ferro: presença ou ausência de ácido ascórbico e presença ou ausência de PtII. Como esperado, a taxa de captação de Fe^{2+} na presença de ácido ascórbico não foi alterada pela adição de PtII em ambas as linhagens **(Figura 4)**. Na ausência de ácido ascórbico, a linhagem *Pb*01 apresentou um ligeiro aumento na taxa de captação de ferro na presença de PtII **(Figura 4A)**. Isso pode ter acontecido por mecanismos ainda não descritos de ativação enzimática ou de compensação ativados pela presença de PtII. Já a linhagem *Pb*18 apresentou uma pequena diminuição na taxa de captação de ferro na presença de PtII, o que sugere que o fungo possua algumas redutases férricas sensíveis à inibição por PtII **(Figura 4B)**.

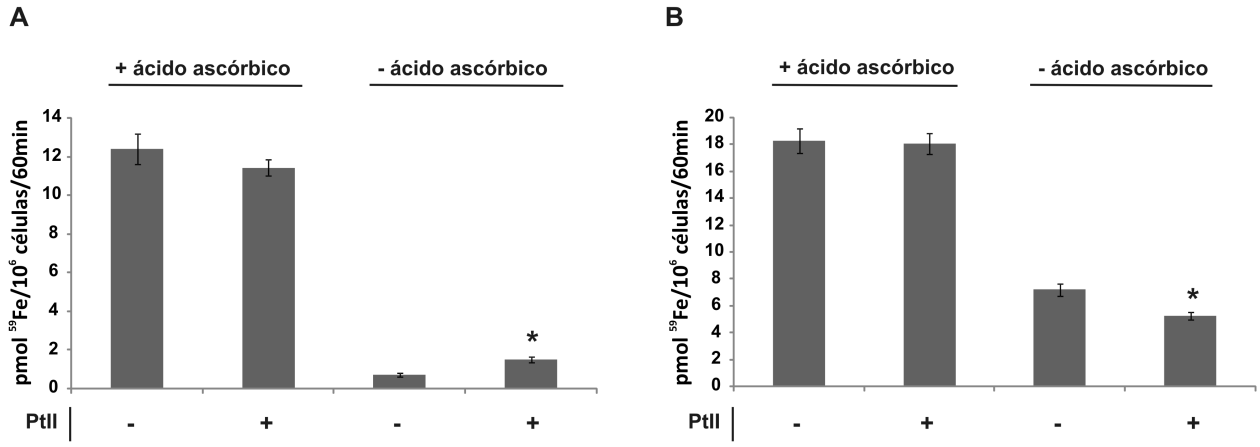

Figura 4. Efeito da adição de um composto de platina sobre a taxa de captação de ferro por *Paracoccidioides* spp. As linhagens *Pb*01 e *Pb*18 foram cultivadas em meio SC suplementado com 200 µM de BPS por 24 h. Após esse período, as células foram coletadas, lavadas e incubadas na presença de ^{59}Fe, com adição (+) ou não (-) de ácido ascórbico e/ou de um composto de platina (PtII), por 60 min. Posteriormente, as células foram novamente coletadas e lavadas, e submetidas a um contador de radiação γ para determinação da velocidade de captação de ^{59}Fe em *Pb*01 **(A)** e em Pb18 **(B)**. Os dados estão apresentados como a média $\pm$ o desvio padrão das triplicatas ou quadruplicatas. *Amostras com aumento ou diminuição estatisticamente significativa ($p < 0,05$) com relação às células cultivadas na ausência de PtII, pelo teste t de Student.

V. MATERIAIS E MÉTODOS DOS RESULTADOS AINDA NÃO PUBLICADOS

V.1. Isolado do fungo e condições de cultivo

Foram utilizados os isolados*Pb*01 (ATCC MYA-826; *Paracoccidioides lutzii*)(TEIXEIRA et al., 2013) e *Pb*18 (ATCC 32069; *Paracoccidioides brasiliensis*, espécie filogenética S1) (CARRERO et al., 2008). Os fungos foram mantidos em meio BHI (infuso de cérebro e coração, Himedia®, Bhaveshwar, Mumbai, India) suplementado com 4% de glicose, a 36 °C para crescimento da forma de levedura. Para os ensaios realizados neste trabalho foram utilizados também os meios quimicamente definidos McVeigh-Morton modificado (MMcM) (RESTREPO; JIMENEZ, 1980) e Sintético Completo (SC) (SHERMAN, 2002), suplementado ou não com 200 μM de BPS ou com 10 μM de $FeCl_3$.

V.2. Ensaio qualitativo de atividade de redutase férrica em meio sólido

As células leveduriformes de *Pb*01 foram crescidas sob filtros de Nylon colocados na superfície do meio BHI sólido. Estes suportes foram transferidos para placas com meio MMcM, contendo 300 μM $FeCl_3$, com ou sem 1 mM de EDTA, como quelante de ferro. Estas placas foram incubadas a 36 °C por 6 h ou por 15 h. Os filtros foram removidos das placas, incubados por 5 min em papel Whatman3MM embebido em tampão citrato (citrato de sódio 50 mM, 5% glicose, pH 6,5) e, então, transferidos para um outro papel Whatman embebido em tampão citrato, contendo 1 mM $FeCl_3$ e 1 mM BPS. Após 5 min, colônias com atividade de redutase férrica pigmentam o filtro de vermelho, pela formação de um complexo BPS[Fe^{2+}].

V.3. Análise de transcritos por qRT-PCR em tempo real

As células leveduriformes de *Pb*01 foram incubadas em MMcM sem adição de ferro e suplementado com baixa concentração de ferro inorgânico (3,5 μM) por diferentes tempos. O RNA total das células foi extraído utilizando o reagente Trizol (Invitrogen™, Life Technologies) e pérolas de vidro, sob vigorosa agitação. Os RNAs obtidos foram utilizados para síntese de cDNAs utilizando-se a enzima transcritase reversa *SuperScript™ II* (Invitrogen™, Life Technologies). Oligonucleotídeos específicos para as sequências codificantes para proteínas possivelmente envolvidas na homeostase de ferro foram construídos. Os níveis de expressão dos transcritos codificantes para essas proteínas foram obtidos por qRT-PCR em tempo real, utilizando-se *SYBR Green PCR master mix* (Applied Biosystems, Foster City, CA) no sistema *StepOnePlus™ Real-Time PCR* (Applied Biosystems Inc.). Os níveis de expressão relativa dos cDNAs utilizados foram

calculados utilizando-se o método de curva padrão para quantificação relativa (BOOKOUT et al., 2006). Os níveis de expressão dos genes de interesse foram normalizados utilizando os valores obtidos para *α-tubulina* (PAAG_01647) de *Paracoccidioides*. Para as análises estatísticas foi aplicado o teste t de Student, com $p < 0,05$.

V.4. Ensaios de captação de ^{59}Fe

Células das linhagens *Pb*01 e *Pb*18 foram cultivadas em meio BHI suplementado com 4% de glicose por 72 h a 36 °C. Após esse período, as células foram coletadas, lavadas e incubadas em meio SC suplementado ou não com 200 μM de BPS, ou com 10 μM de $FeCl_3$ por 24 h a 36 °C. Posteriormente, as células foram coletadas e lavadas uma vez com 1 mM de EDTA e duas vezes com tampão de captação (2% glicose AnalaR; 0,1 M tampão MES; 20 mM citrato de sódio; pH 6,0). Após as lavagens, as células foram incubadas em tampão de captação por 15 min a 36 °C e contadas na presença do corante Trypan Blue para a estimativa do número de células viáveis. Posteriormente, foi adicionado ao sistema de captação 5 μM de ^{59}Fe acompanhado ou não da adição de 20 mM de ácido ascórbico e/ou de 100 μM de Cl_4K_2Pt (PtII). Uma amostra foi imediatamente coletada e depositada em tampão *Quench* (37,5 mM ácido succínico; 62,5 mM Tris; 12,8 mM EDTA; pH 6,0) para servir como controle de aferição da radiação (tempo 0) e o restante do material foi incubado por 60 min. Após esse período, as células foram coletadas em tampão *Quench*, lavadas 3 vezes com esse mesmo tampão e levadas para a leitura em um contador de radiação γ. A coleta das amostras foi feita em triplicata ou quadruplicata. Os dados obtidos foram plotados em gráficos de barras como a média das triplicatas ou quadruplicatas $\pm$ o desvio padrão das mesmas. Para as análises estatísticas foi aplicado o teste t de Student, com $p < 0,05$.

VI. DISCUSSÃO

Dados *in silico* apontaram que *Paracoccidioides* spp. deve possuir um sistema de captação de ferro pela via redutiva, que seria composto por uma ou mais redutases férricas, uma ferroxidase e uma permease de ferro e zinco. Após atingir o interior da célula, o ferro teria dois destinos principais. (i) A mitocôndria, onde seria transportado por uma proteína homóloga a Mrs3/4 e suas concentrações seriam monitoradas pela proteína Yfh1, homóloga da frataxina humana. (ii) Ou o vacúolo, onde o excesso de ferro do citoplasma seria transportado pela proteína Ccc1 e estocado no interior desta organela. Além disso, essas análises apontaram que o fungo possui uma redutase férrica extracelular dependente de glutationa (Ggt1) (SILVA et al., 2011).

Neste trabalho, uma atividade de redutase férrica na superfície de *Paracoccidioides* sp. foi demonstrada. Além disso, a atividade de redutase férrica dependente de glutationa já foi comprovada no sobrenadante de cultura de leveduras de *Paracoccidioides* sp. na presença de Fe^{3+} e glutationa, como cofator (ZARNOWSKI; WOODS, 2005). A enzima responsável por essa atividade deve ser secretada e pode ajudar o fungo a obter ferro de sideróforos e outros substratos contendo Fe^{3+}, como a transferrina, como já demostrado para *Histoplasma capsulatum* (TIMMERMAN; WOODS, 2001). Esses dados indicam que o sistema de redutase férrica não só existe em *Paracoccidioides* spp., como também é funcional. As redutases férricas deste fungo seriam reguladas por ferro, uma vez que o cultivo de *Paracoccidioides* sp. em meio de cultura sem adição de ferro aumenta a expressão dos genes codificantes dessas enzimas em duas a três vezes, em comparação ao fungo cultivado em meio padrão contendo ferro (BAILÃO et al., 2012).

As ferroxidases de *Paracoccidioides* spp. não possuem resíduos já descritos como essenciais para a atividade de ferroxidase (BONACCORSI DI PATTI et al., 2000) e são todas classificadas como lacases pelo banco de dados de lacases e multicobre oxidases disponível *online* (http://www.lcced.uni-stuttgart.de/cgi-bin/LccED1.2/index.pl) (SIRIM et al., 2011). Porém já foi reportada a atividade de ferroxidase para uma lacase de *C. Neoformans* (LIU; TEWARI; WILLIAMSON, 1999) e a existência de ferroxidases não convencionais já foi sugerida (LARRONDO et al., 2003; LARRONDO et al., 2004; CANESSA; LARRONDO, 2013). As ferroxidases de *Paracoccidioides* spp. devem atuar em parceria com permeases de zinco e ferro, já que esse fungo não apresenta em seu genoma homólogos de permeases de ferro de alta afinidade (SILVA et al., 2011). Este dado corrobora a hipótese de que Zrt1 e Zrt2 fariam a captação de ferro em *Paracoccidioides* spp. (BAILÃO et al., 2006; BAILÃO et al., 2007). A expressão dos genes

lacIV e *zrt2* parece ser coordenada e induzida pela escassez de ferro, sugerindo que as proteínas codificadas por esses genes possam atuar em conjunto para a captação desse metal em *Paracoccidioides* spp.

Um dos destinos metabólicos do ferro é a mitocôndria. Com a diminuição da disponibilidade desse micronutriente, foi observado um aumento da expressão do gene *mrs3/4* de *Paracoccidioides* sp., provavelmente porque o ferro é bastante importante para a atividade mitocondrial (FROSCHAUER; SCHWEYEN; WIESENBERGER, 2009) e precisa ser eficientemente captado para o interior da mitocôndria. Já foi demonstrado que a atividade de Mrs3 e Mrs4 é estimulada pela escassez de ferro em *S. cerevisiae* (FROSCHAUER; SCHWEYEN; WIESENBERGER, 2009). O outro principal destino intracelular do ferro é o vacúolo. A expressão do gene *ccc1* de *Paracoccidioides* sp. diminuiu durante a privação de ferro. Em *S. cerevisiae* já foi demonstrado que, durante a escassez desse metal, há uma diminuição no número de transcritos codificantes para Ccc1, prevenindo a estocagem de ferro e permitindo sua disponibilidade a processos ferro-dependentes (PUIG; ASKELAND; THIELE, 2005).

A taxa de captação de Fe^{2+} em *Paracoccidioides* spp. foi maior em leveduras cultivadas na presença do quelante de ferro BPS. Esse resultado indica que a privação de ferro enfrentada pelo fungo pode induzir à exposição de moléculas envolvidas com a captação do metal na superfície do micro-organismo, o que estimularia a captação de Fe^{2+}. O contrário deve acontecer quando o meio de cultura do fungo é suplementado com ferro, como demonstrada pela menor taxa de captação de Fe^{2+} de células cultivadas nessa condição. Quando a taxa de captação de ferro dependente de redutases férricas foi avaliada, a captação de ^{59}Fe foi detectada apenas em *Pb*18 cultivado na presença de BPS. Resultados similares já foram observados em *S. cerevisiae* (EIDE et al., 1992). O que foi surpreendente foi a não detecção da captação de ^{59}Fe dependente de redutase férrica em *Pb*01 cultivado na presença de BPS. O que se pode sugerir é que o sistema de redutases férricas de *Pb*01 não é eficiente, mas essa hipótese ainda precisa ser investigada.

Foi observada uma leve inibição da captação de ferro pela platina em *Pb*18 e um leve aumento da captação em *Pb*01. Contrariamente, em *S. cerevisiae* compostos contendo Pt^{2+} (PtII) inibem severamente a atividade de redutase férrica e, consequentemente, a captação de Fe^{3+}(EIDE et al., 1992). Parece que essa inibição acontece preferencialmente sobre a enzima Fre1 (HASSETT; KOSMAN, 1995), a principal redutase férrica de *S. cerevisiae* (DANCIS et al., 1992). Apesar de *Paracoccidioides* spp. possuir homólogos para *fre1* (SILVA et al., 2011), não foi observada a

expressão desse gene em *Pb*01 nas condições testadas neste trabalho (dados não mostrados), sugerindo que esse gene não é funcional em *Paracoccidioides* spp. Essa hipótese explicaria o pequeno efeito da PtII sobre a captação de Fe^{3+} neste fungo.

O micronutriente ferro é essencial para todos os eucariotos. Dessa maneira, desvendar os mecanismos e a cinética de captação desse metal em fungos patogênicos é de fundamental importância. Estratégias que inibam seletivamente a captação de ferro pelo patógeno podem ser utilizadas em terapias conjuntas com antifúngicos para eliminação do invasor (KRONSTAD; CADIEUX; JUNG, 2013). Mais estudos precisam ser realizados para definir melhor os mecanismos de captação de ferro em *Paracoccidioides* spp. para que moléculas envolvidas nesse sistema possam ser utilizadas como possíveis alvos de vacinas ou drogas antifúngicas.

Capítulo 4

Considerações finais

I. CONSIDERAÇÕES FINAIS

Paracoccidioides spp., um fungo termodimórfico, patogênico humano, é capaz de utilizar a hemoglobina ou grupos heme como fonte de ferro, como demonstrado por ensaios de crescimento em placa e com a utilização de uma protoporfirina auto-fluorescente. Porém o crescimento do fungo na presença de transferrina ou ferritina como fontes de ferro sugere que o fungo utilize várias estratégias para a captação desse micronutriente essencial durante a infecção. Já foi sugerido para outros fungos que a captação de transferrina é dependente da via redutiva. *Paracoccidioides* spp. parece apresentar uma via redutiva ativa, como demonstrado por ensaios de atividade de redutase férrica e de captação de ^{59}Fe, o que sugere que este fungo também possa utilizar o ferro da transferrina por essa via.

Para conseguir captar o ferro da hemoglobina, inicialmente, o fungo precisar romper as hemácias e, posteriormente, promover a internalização do metal. Foi demonstrado que o fungo produz um fator hemolítico através de ensaios *in vitro* utilizando hemácias de carneiro. Além disso, análises *in silico* apontaram a existência de uma família de receptores de superfície em *Paracoccidioides* spp. que poderia ser capaz de promover a ligação e a internalização da hemoglobina. Os genes codificantes para esses receptores de *Pb*01 foram investigados e demonstraram ser ativados em baixas concentrações de ferro e na presença de hemoglobina.

Quando o proteoma de *Pb*01 cultivado na presença de hemoglobina foi investigado e comparado ao proteoma do fungo cultivado na presença de ferro inorgânico, os resultados apontam que *Paracoccidioides* spp. possa utilizar hemoglobina não só como fonte de ferro, mas também de nitrogênio, enxofre e porfirina, internalizando toda a molécula de hemoglobina e não somente o ferro. Essa captação de hemoglobina seria independente da via redutiva, já que o ferro não seria liberado extracelularmente, e ocorreria por endocitose, como já foi descrito e sugerido para outros fungos.

Um dos possíveis receptores de hemoglobina de *Pb*01 foi investigado, Rbt5, e demonstrou ser uma proteína GPI ancorada na superfície do fungo, com capacidade de ligação à hemina, hemoglobina e protoporfirina e que pode atuar como antígeno de superfície. Uma linhagem silenciada para o gene codificante de Rbt5 foi eficientemente gerada e apresentou menor sobrevivência em modelos de infecção utilizando macrófagos e camundongos, indicando que Rbt5 pode ser um importante fator de virulência, contribuindo para o estabelecimento ou a manutenção da infecção, ou que esta proteína interfere na estimulação da resposta imunológica dos macrófagos

para matar as células fúngicas internalizadas. Como a linhagem silenciada apresentou crescimento similar à linhagem selvagem na presença de hemoglobina, outras moléculas devem atuar na superfície de *Paracoccidioides* spp. para captar essa molécula do hospedeiro, como já apontavam as análises *in silico*.

Apesar da via redutiva não parecer atuar na captação de hemoglobina, ela deve ser ativada também durante a infecção, já que ela pode ser importante para a captação de ferro de outras fontes do hospedeiro. Um modelo para a via redutiva de *Paracoccidioides* spp. foi sugerido. O fungo não apresenta algumas proteínas clássicas, como transportadores de alta afinidade e ferroxidases, porém essas moléculas podem ter tido suas funções substituídas por outras, como transportadores de ferro e zinco e ferroxidases não convencionais, cujos genes codificantes foram identificados *in silico* no genoma de *Paracoccidioides* spp.

As redutases férricas identificadas *in silico* em *Pb*01 foram investigadas e apresentaram-se funcionais e a expressão dos genes codificantes para essas enzimas parece ser regulada positivamente na ausência de ferro, indicando que elas participam de um sistema de captação de ferro de alta afinidade. Os genes codificantes para proteínas possivelmente envolvidas com a oxidação e o transporte de ferro para dentro da célula e para dentro da mitocôndria também têm sua expressão regulada positivamente na ausência de ferro. Contrariamente, a expressão do gene que codifica para um possível transportador de ferro vacuolar é regulada negativamente na ausência do metal, uma vez que não é necessário estocar ferro durante a escassez do mesmo.

A avaliação da taxa de captação de ^{59}Fe revelou que *Paracoccidioides* spp. capta Fe^{2+}, já em sua forma reduzida, mais eficientemente que Fe^{3+} e que a via redutiva só é ativada nesse fungo durante a privação do metal. Além disso, as redutases férricas desse fungo são pouco afetadas pela platina e não foi observada a expressão do gene *fre1* de *Pb*01 nas condições utilizadas neste trabalho, o que indica que as enzimas funcionais de *Paracoccidioides* spp. não são homólogas à Fre1 de *S. cerevisiae* e, portanto, não são sensíveis à platina.

Em conjunto, esses resultados sugerem que *Paracoccidioides* spp., assim como outros fungos estudados, apresentam várias estratégias para adquirir ferro. (i) Captação de hemoglobina mediada por receptores de superfície, como a Rbt5. (ii) Via redutiva, contendo redutases férricas, ferroxidases não convencionais e transportadores de ferro e zinco. E (iii) produção e captação de sideróforos (SILVA-BAILÃO et al., fase final de redação).

II. PERSPECTIVAS

As perspectivas que surgiram a partir dos resultados deste trabalho são:

- Estabelecer a taxa de redução de ferro em *Paracoccidioides* spp., utilizando um ensaio colorimétrico;
- Investigar a expressão de genes possivelmente envolvidos com a via redutiva em *Paracoccidioides* spp. nas mesmas condições utilizadas para a verificação da taxa de captação de ^{59}Fe, por RT-PCR em tempo real;
- Extrair peptídeos de superfície das leveduras de *Paracoccidioides* sp. por digestão com tripsina;
- Incubar as moléculas obtidas com uma resina de agarose ligada à hemina;
- Identificar peptídeos ligantes de hemina por cromatografia líquida de alta performance em nano-escala acoplada à espectrometria de massas com aquisições de dados independentes (nanoUPLC-MS^E);
- Selecionar uma proteína identificada por nanoUPLC-MS^E para produção da molécula recombinante e, posteriormente, de anticorpos policlonais;
- Confirmar a localização da proteína selecionada na levedura utilizando os anticorpos policlonais produzidos e microscopia eletrônica de transmissão;
- Confirmar a ligação da proteína selecionada à hemina utilizando a proteína recombinante e a resina hemina-agarose;
- Investigar as possíveis funções da proteína selecionada construindo uma linhagem de *Paracoccidioides* sp. silenciada para o gene codificante dessa proteína utilizando a tecnologia do RNA antisentido e a transformação mediada por *Agrobacterium tumefaciens*.

III. REFERÊNCIAS BIBLIOGRÁFICAS

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