Published in final edited form as:

J Proteome Res. 2012 January 1; 11(1): 189–205. doi:10.1021/pr2005296.

Proteomic profiling of the influence of iron availability on *Cryptococcus gattii*

Juliana Crestani[†], Paulo Costa Carvalho^{†,§}, Xuemei Han[‡], Adriana Seixas[†], Leonardo Broetto[†], Juliana de Saldanha da Gama Fischer^{§,£}, Charley Christian Staats^{†,□}, Augusto Schrank^{†,□}, John R Yates III^{‡,¶}, and Marilene Henning Vainstein^{†,□,*,#}

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

[‡]Laboratório de Toxinologia, Instituto Oswaldo Cruz, Rio de Janeiro, Brasil

[§]Centro de Desenvolvimento Tecnológico em Saúde (CDTS), Fundação Oswaldo Cruz, Rio de Janeiro, Brasil

[¶]Department of Chemical Physiology, The Scripps Research Institute, La Jolla, California, USA

[£]Laboratório de Genômica funcional e Bioinformática, Instituto Oswaldo Cruz, Rio de Janeiro, Brasil

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

Abstract

Iron is essential and ubiquitous in living organisms. The competition for this micronutrient between the host and its pathogens has been related to disease establishment. *Cryptococcus gattii* is an encapsulated yeast that causes cryptococcosis mainly in immunocompetent individuals. In this study, we analyzed the proteomic profile of the *C. gattii* R265 Vancouver Island isolate under iron-depleted and –replete conditions by Multidimensional Protein Identification Technology (MudPIT) and by 2D-GE. Proteins and key mechanisms affected by alteration of iron levels such as capsule production, cAMP-signaling pathway, response to stress, and metabolic pathways related to mitochondrial function were identified. Our results also show both proteomic methodologies employed to be complementary.

Keywords

Cryptococcus gattii R265; Proteomics; 2D-LC-MS/MS; MudPIT; 2D-GE; iron

1. INTRODUCTION

Cryptococcus gattii is a yeast pathogen that causes cryptococcosis commonly in immunocompetent humans and less frequently in immunosuppressed individuals.^{1–3}

*Corresponding author: Marilene Henning Vainstein, Centro de Biotecnologia, Universidade Federal do Rio Grande do Sul, Av. Bento Gonçalves 9500, 43421, Caixa Postal 15005, Porto Alegre, RS 91501-970, Brazil. mhv@cbiot.ufrgs.br, Phone: +55 51 3308 6060. Fax: +55 51 3308 7309.

#JRY and MHV share senior authorship on this article.

ASSOCIATED CONTENT

Supporting Information Available

Supplemental Tables S1 – S5 and Supplemental Figure S1 are available free of charge via the Internet at <http://pubs.acs.org>.

Cryptococcosis is acquired by the inhalation of airborne propagules from an environmental source; these invade the lungs and cause a primary pulmonary infection. This pulmonary infection disseminates through the bloodstream and infects the central nervous system causing meningoencephalitis, which is fatal if untreated.⁴⁻⁶

C. gattii caused a cryptococcosis outbreak in Vancouver Island, British Columbia, Canada. Genetic studies demonstrated that this outbreak was caused by distinct genotypes of *C. gattii*, VGI and VGII.⁷ The VGII genotype is divided in two subtypes; a major hypervirulent VGIIa, and a minor lower virulent VGIIb.⁷ Substantial morbidity and mortality rates were observed for infections caused by the hypervirulent subtype of *C. gattii*.⁸⁻¹¹ This scenario has stimulated detailed investigations on this organism.¹²⁻¹⁵

C. gattii and its closely related species *C. neoformans* share virulence determinants, including the production of a polysaccharide capsule, the synthesis of melanin, the production of extracellular enzymes and the ability to grow at 37°C.⁵ However, in *C. gattii*, only a few genes such as those for phospholipase B, superoxide dismutase, a transcription factor *STE12a*, and protein kinases, have been studied in connection with pathogenesis.¹⁶⁻²² Ma and colleagues²³ proposed that the VGIIa and VGIIb *C. gattii* subtypes, have an altered mitochondrial function regulation, leading to an increased capacity to survive and proliferate in macrophages and consequently the hypervirulent phenotype. Mitochondrial functions influence important cellular processes including iron metabolism. For example, certain heme synthesis reactions occur in mitochondrion, as well as the formation of iron-sulfur (Fe/S) cluster which are essential for many metabolic enzymes.^{24,25} The iron metabolism has been described in *C. neoformans*,²⁶⁻³² showing important roles in the proliferation of *C. neoformans* and establishment of cryptococcosis.^{28,33} For *C. gattii*, however, limited information are available regarding its iron metabolism.³⁴

To access the possible roles of the iron metabolism in the iron regulation of virulence in *C. gattii* we compared the proteomic profile of the representative strain of the major VGIIa subtype of *C. gattii* from the Vancouver outbreak under iron-depleted and replete conditions by using Multidimensional Protein Identification Technology (MudPIT)³⁵⁻⁴² and 2D-GE. Our results identified proteins involved in iron pathways in *C. gattii* that were then compared with the ones from *C. neoformans*.^{29,31,43} In addition, we found both proteomic techniques to be complementary.

2. MATERIALS AND METHODS

2.1. Fungal strain and growth conditions

The strain R265 was previously identified by Kidd *et al.*⁷ and it was a gift from Dr Wieland Meyer from the culture collection of Molecular Mycology Research Laboratory Westmead Hospital. The strain was grown in 10 ml of Yeast Peptone Dextrose (YPD) broth (yeast extract 1%, peptone 1% and glucose 2%) prior to cultivation in 100 ml of Yeast Nitrogen Base broth (YNB, Becton, Dickinson and Company, Sparks, USA) for 24 h. In order to evaluate the effects of iron on the global protein expression, cells (5×10^7 cells.ml⁻¹) were transferred to 50 ml of Low-Iron medium (LIM, according Jacobson *et al.*³²) and of Iron-replete medium (LIM+Fe, added of 100 µM FeHEDTA, Sigma Chemical Co., Saint Louis, USA) for 12 h as described before.³⁰⁻³² All cultures were grown at 37 °C with shaking (200 rpm).

2.2. Preparation of protein extracts

Cells were collected by centrifugation at 7,500 ×g for 10 min, lyophilized and then disrupted with mortar and pestle in liquid nitrogen to a fine powder. The samples were suspended in

buffer with protease inhibitors (50 mM Tris-HCl pH 7.5, 1 mM EDTA, 1 mM PMSF, 50 μ M TPCK, 5 mM Iodoacetamide). Proteins were solubilized by vortexing the suspensions for 5 min, followed by centrifugation at 10,000 g for 20 min. Supernatants were collected and preserved at -20°C . Remaining cell debris were suspended in the same buffer, followed by vortexing (5 min) and sonication (25 Hz in a VC601 Sonics and Materials Inc. sonicator) in an ice bath for three 30 sec cycles with 1 min intervals. Supernatant was collected after centrifugation, pooled with the first supernatant and stored at -80°C . Protein concentration was determined using the Bradford assay (Bio-Rad, Hercules, CA).

2.3. Sample preparation and MudPIT

Approximately 100 μ g *C. gattii* protein extracts were suspended in digestion buffer (8 M urea, 100 mM Tris-HCl pH 8.5). Proteins were reduced with 10 mM dithiothreitol at 37°C for 30 min and alkylated with 15 mM iodoacetamide at room temperature in the dark for 20 min. After addition of 1 mM CaCl_2 (final concentration) the proteins were digested with 2 μ g of trypsin (Promega, Madison, USA) by incubation at 37°C during 16 h. Proteolysis was stopped by adding formic acid to a final concentration of 5%. Samples were centrifuged at $18,000 \times g$ for 30 min and the supernatant was collected and stored at -20°C . The conditions used for 2D-LC-MS/MS were as described by Washburn *et al.*⁴⁴ The flow rate at the tip of the biphasic column was 100 nl/min when the mobile phase composition was 95% H_2O , 5% acetonitrile and 0.1% formic acid. The ion trap mass spectrometer (Finnigan LTQ-XL, Thermo, San Jose, CA) was set to the data-dependent acquisition mode with dynamic exclusion turned on. One MS survey scan was followed by nine MS/MS scans and 12 salt steps were performed. Mass spectrometer scan functions and HPLC solvent gradients were controlled by the Xcalibur data system (Thermo, San Jose, CA). Three biological replicates and two technical replicates were analyzed for both *C. gattii* R265 culture conditions (LIM and LIM+Fe).

2.4. Interpretation of MS/MS Data Sets

Tandem mass spectra were extracted from the raw files using RawExtract 1_9_3⁴⁵ and were searched with SEQUEST⁴⁶ against the *C. gattii* R265 strain database plus common contaminant proteins. All sequences were downloaded as FASTA-formatted files from the Broad Institute protein database, released on August 5, 2009 (http://www.broadinstitute.org/annotation/genome/cryptococcus_neoformans_b/MultiDownloads.html). The peptide mass search tolerance was set to 3 Da. Carboxymethylation was considered as a static modification. No enzymatic cleavage conditions were imposed on the database search, so the search space included all candidate peptides, whose theoretical mass fell within the 3 Da mass tolerance window, regardless of their tryptic status. The validity of peptide/spectrum matches was assessed in DTASelect⁴⁷ according to the SEQUEST cross-correlation score (XCorr) and the SEQUEST normalized difference in cross-correlation score (DeltaCN). The search results were grouped by charge state (+1, +2, and +3) and tryptic status (Fully tryptic, half-tryptic, and non-tryptic), resulting in nine subgroups. In each of the subgroups, the distribution of XCorr and DeltaCN values for the direct and decoy database hits were obtained and the two subsets were separated by quadratic discriminant analysis. Outlier points in the two distributions (for example, matches with very low XCorr but very high DeltaCN) were discarded. As the full separation of the direct and decoy subsets is not generally possible, the discriminant score was set such that a false discovery rate of 1% was determined based on the number of accepted decoy database peptides. This procedure was independently performed on each data subset, resulting in a false-positive rate independent of tryptic status or charge state. In addition, a minimum sequence length of 7 amino acid residues was required, and each protein on the list was supported by at least two peptide identifications. These additional requirements, especially the latter, resulted in the elimination of most decoy database and

false-positive hits, as these tended to be overwhelmingly present as proteins identified by single peptide matches. After this last filtering step, the estimated false discovery rate was reduced to below 1%.

2.5. PatternLab for Proteomics analysis

PatternLab^{48, 49} was used to pinpoint differentially expressed proteins found in both biological conditions and the ones identified in a single condition. A spectral count database (DB) was firstly obtained by using PatternLab to parse the DTASelect files resulting from the *C. gattii* LIM and *C. gattii* LIM+Fe biological triplicates to the PatternLab's native data format (sparse matrix and index files). PatternLab's TFold module⁵⁰ was used for selecting proteins found in both conditions that were considered as differentially expressed. The spectral counts from each run were normalized according to the Row Sigma normalization.⁴⁹ Proteins that were not detected in at least 9 out of the 12 assays (i.e., necessarily detected in both conditions) were not considered in the TFold analysis. Proteins whose average of the spectral counts had an absolute fold change greater than 2.5 and *p*-value of less than 0.05 according to the t-test were considered as differentially expressed. The fold change cutoff was established by iteratively increasing the cutoff as to have the *p*-values from all differentially expressed proteins satisfy the BH false discovery estimator criterion of 0.05.⁵¹

PatternLab's Approximately Area Proportional Venn Diagram (AAPVD) module³⁵ was used for pinpointing proteins uniquely identified in a condition. However, we must note that even though a protein was not identified for a condition it could still be present, but under the detection threshold. For the AAPVD analysis, proteins identified in only one of the replicates were discarded.

2.6. Gene Ontology (GO) annotation and KEGG analysis

The Blast2GO tool (<http://www.blast2go.org>) was used to obtain functional categories of proteins.^{52, 53} This tool assigns the Gene Ontology (GO) terms based on the BLAST definitions. This was accomplished by submitting the FASTA sequences of the identified proteins to the Blast2GO platform, where they were compared against NCBI databases (http://www.ncbi.nlm.nih.gov/staff/tao/URLAPI/remote_blastdblist.html). Briefly, Blast2GO used BlastP, using the default parameters, to identify the similar proteins that had GO annotations. The data generated by the Blast2GO analysis were represented at the GO levels 2, 3, and at multilevels, illustrating the general functional categories according biological process and molecular functions. KEGG pathways⁵⁴ were assigned based on the GO annotation.

2.7. Two-dimensional Gel Electrophoresis

Lyophilized proteins fractions (ca. 1.2 mg) were submitted to ReadyPrep 2-D Cleanup kit (Bio-Rad, Hercules, CA) according to the manufacturer's recommendations. The final pellet was suspended in 300 µl of isoelectric focusing buffer (9 M Urea, 4% CHAPS, 1% Dithiothreitol (DTT) and 0.2% Ampholytes pH 3–10 (Bio-Rad, Hercules, CA). The 17 cm immobilized pH gradient strips (pH 4–7, Bio-Rad, Hercules, USA) were passively rehydrated for 16 h. Isoelectric focusing was performed in a Protean IEF cell system (Bio-Rad, Hercules, USA) with a gradual increase in voltage (250 V for 15 min, followed by 10,000 V until total of 60,000 Vh) with a maximum current setting of 50 µA per strip at 20 °C. Focused strips were either used immediately or were stored at –80 °C until use. Before executing the second dimension the strips were soaked (15 min) with 10 ml of equilibration buffer (30% glycerol, 6 M Urea, 1% DTT and a trace of Bromophenol blue) followed by incubation (15 min) in a similar solution, except for the replacement of DTT by 4% iodoacetamide. For second dimension vertical SDS-PAGE 12% gels were run using

PROTEAN II xi 2D Cell (Bio-Rad, Hercules, USA) at a constant current 50 mA for about 7 h. Proteins were visualized by Coomassie Brilliant Blue G 250 (Sigma Chemical Co., Saint Louis, USA).

Gels were scanned using the Imager Scanner (Amersham Biosciences, Uppsala, Sweden) and analyzed with the PDQuest 2-D Gel Analysis Software version 8.0.1 (Bio-Rad, Hercules, USA). Selected spots from individual gel images were first matched to at least two other replicate gels. We considered differentially expressed proteins between two experimental conditions (LIM and LIM+Fe) as the ones having $p < 0.01$ according to the Student's t-test. Fold change 2.0 was determined per group. After automatic spot detection, manual editing was performed on all spots to avoid incorrect match detected by the software. Experiments were performed with three biological and technical replicates.

2.8. In-gel digestion and LC-MS/MS peptide analysis

Protein spots were excised from replicate gels and chopped into $\sim 1 \times 1$ mm pieces after electrophoresis of extracts from both experimental conditions (LIM and LIM+Fe). In-gel digestion was performed according Niessen *et al.*⁵⁵ Recovered peptides were loaded onto a single-phase column containing C18 resin as described.⁵⁶ The chromatographic separation consisted of an initial wash step with buffer A (5% ACN: 0.1% formic acid, v:v) for 10 min, and subsequent elution of peptides with a linear gradient from 0 – 100% buffer B (90% ACN: 0.1 % formic acid, v:v) over 110 min. The HPLC column eluent was directly transferred into the electrospray ionization source of an ion trap mass spectrometer (Finnigan LTQ-XL, Thermo, San Jose, CA). MS/MS dataset interpretations were performed as described above.

2.9. Peptidase activity assays

Protease and metalloprotease activity were tested upon fluorogenic substrates, including N-*t*-Boc-Gly-Arg-Arg-MCA (*t*-Boc, *tert*-Butyl carbamates; MCA, 7-amino-4-methylcoumarin) and N-*t*-Boc-Ile-Glu-Gly-Arg-MCA (Sigma Chemical Co., Saint Louis, USA). All substrates were at a final concentration of 2 μ M in a total assay volume of 100 μ l. Kinetics of hydrolysis was monitored at 11 s-intervals by fluorimetry in a SpectraMax M2-e Microplate Reader (Molecular Devices Corporation, California, USA). The wavelength pair for excitation and emission was 370/ 460 nm.⁵⁷ Protein samples from *C. gattii* R265 cultures in LIM and LIM+Fe (10 μ l, 20 μ g of protein) conditions were incubated for 10 min at 37°C in Tris-HCl saline buffer, pH 7.2, in the presence or absence of specific protease inhibitors 10 mM EDTA; 2 μ M pepstatin A; 10 μ M L-trans-epoxysuccinyl-L-leucylamido(4-guanidino)-butane (E-64) and, 100 μ M phenylmethanesulfonylfluoride (PMSF), prior substrate addition. Peptidase activity was given in nMoles of MCA calculated using a standard curve.

2.10. Western blot

For western blots, 20 μ g of *C. gattii* proteins were separated by SDS-PAGE and transferred to a polyvinylidene fluoride (PVDF) membrane. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (37 kDa) was detected with polyclonal antisera against the recombinant GAPDH from *Paracoccidioides brasiliensis*.⁵⁸ Protein expression levels were quantified with the ImageJ software (NIH, Maryland, USA).

2.11. Reductase activity

Cell surface reductase activity was measured by using the TTC (tetrazolium chloride) assay as described previously.^{30, 59}

3. RESULTS

3.1. MudPIT identifications

The results obtained using MudPIT include a total of 1080 non-redundant proteins identified from LIM and 1135 from LIM+Fe conditions. (Figure 1C and Supplementary Table S2, Supporting Information). Venn diagrams displaying the distribution, overlap, and reproducibility of the identified proteins for the three biological replicates of each condition are displayed in Figure 1A and B. The proteins identified with two or more peptides are listed in Table S1- Supporting Information.

3.2. Gene Ontology Analysis

A total of 725 proteins were found in at least 3 or more replicates; these proteins were selected for a GO analysis. Of these proteins, forty eight were exclusively identified when *C. gattii* was grown under iron deprivation (LIM); 16 were found to be uniquely expressed under iron replete, and the remaining were common to both conditions (Figure 1D).

GO annotation⁶⁰ provided a broad view of the *C. gattii* response to different conditions of iron availability (Figure 2). Ten heme-binding proteins were identified in response to iron alteration, which suggest that *C. gattii* may also use heme as iron sources, as *C. neoformans*.²⁸ Many proteins linked to structural constituent of ribosome (60 proteins), antioxidant activity (12 proteins) and oxidoreductases activities (36 proteins) were observed in our analysis, which were also evidenced in a previous study in *C. neoformans*.³¹ These results, together with a more complete classification in the GO categories are found on Supplementary Table S3 (Supporting Information).

3.3. Analysis of the Hypothetical proteins

A total of 183 proteins (about 23% of the all proteins detected in at least 3 replicates) were identified as hypothetical or conserved hypothetical proteins; these were treated separately as to not compromise the confidence in our global analysis. Their sequences were “BLASTed” against NCBI non-redundant databases and the result identified 30 of these proteins to have a high similarity with proteins from other closely related organisms: *C. neoformans* var. *neoformans* JEC21 and *C. neoformans* var. *grubii* (Table 1). Among the BLAST results we point out: glutamate synthase, isoprenoid biosynthesis, and cytochrome c oxidase. These proteins are related to biosynthetic pathways such as glutamate and nitrogen metabolism, and electron transport chain; they require iron as a cofactor. Proteins whose expression was altered by iron availability also included heat shock proteins (HSP) 12, 60, 70 and 90. Although these molecules still do not have an established role on cryptococcal pathogenesis, their counterparts in microbial pathogens are known to regulate virulence and / or immunological mechanisms.^{61, 62} The 14-3-3 protein, which was described as an immunodominant protein influencing early pathogenic mechanisms in cryptococcosis, was also affected.⁶² One of the proteins with similarity to HSP70 (CNBG_3266) (Table 1), was found to be upregulated (2.6-fold) in response to the LIM condition in our differential expression analysis.

The remaining proteins matched with hypothetical or conserved hypothetical proteins from *Cryptococcus*. Results from sequence analysis and gene ontology annotation of all hypothetical proteins are available in Supplementary Table S4 (Supporting Information).

3.4. The Kyoto Encyclopedia of Genes and Genomes (KEGG) Pathway Analysis

In order to identify pathways affected by iron in the culture medium we mapped the MudPIT results to the KEGG pathways database.⁵⁴ Ninety six different pathways were linked to proteins that were identified in both experimental conditions (LIM and LIM+Fe). On the

other hand, proteins related to 15 pathways were exclusively detected in cultures of *C. gattii* in LIM. Threonine ammonia-lyase, a protein linked to four distinct pathways (biosynthesis of secondary metabolites; glycine, serine and threonine metabolism; valine, leucine and isoleucine biosynthesis; metabolic pathways) was only detected as a response to the incubation of *C. gattii* in LIM+Fe (Table S5, Supporting Information). A considerable number of the detected proteins were related to pathways linked to mitochondrial functions, including the citric acid (TCA) cycle, glycolysis/ gluconeogenesis, pentose phosphate pathway and oxidative phosphorylation. Indeed, many iron proteins and iron cofactors are related to mitochondrial activity.

3.5. Analysis of differentially expressed proteins

The semi-quantitative analysis of protein expression after growth of *C. gattii* in LIM or LIM +Fe was performed and a TFold comparison pinpointed 39 differentially expressed proteins. Of these, 12 and 27 proteins were upregulated after fungal growth in LIM (Table 2) and LIM+Fe (Table 3), respectively.

We found a peptidase related to the family of zinc-metalloproteinases to be the protein with the highest differential expression ratio (7.2 fold upregulated) after growth of *C. gattii* in LIM. Another peptidase, extracellular elastinolytic metalloproteinase, was 6.7-fold upregulated when the fungus was cultivated in LIM. These results were validated by peptidase activity assays. In all, fungal extracts obtained from cells grown in LIM showed an increased total protease activity when compared with extracts from cultures of *C. gattii* under iron availability (Figure 3). These results are in agreement with the augmented protease activity in fungal extracts obtained from cells grown in LIM (Figure 3).

Among the proteins related to metabolic processes highlighted in our comparative analysis, we point out the overexpressed enzymes in *C. gattii* grown under iron deprivation: 6-phosphogluconolactonase (5.6-fold), D-lactaldehyde dehydrogenase (3.8-fold), 3-hydroxyacyl-CoA-dehydrogenase (3.6-fold) and GAPDH (2.7-fold). The latter has been implicated in multiple functions such as glycolysis, transcription activation, initiation of apoptosis, and vesicle shuttling from endoplasmic reticulum (ER) to Golgi.^{63, 64} The enzyme 6-phosphogluconolactonase has been described in participating in the oxidative phase of the pentose phosphate pathway, which is involved in the response to oxidative conditions, through the generation of NADPH, that serves as an electron source for reductases and antioxidants.⁶⁵ These results lead us to speculate that iron deprivation may result in oxidative damage response in *C. gattii*, which is commonly observed in other models.^{65, 66}

It has been well-established that iron deprivation regulates the expression of virulence factors in *C. neoformans*, such as capsule formation and melanin production.^{26, 28, 43, 67} Our differential expression analysis showed an UDP-glucose dehydrogenase and a glucose-methanol-choline (GMC) oxidoreductase to be upregulated (2.9-fold and 6.9-fold, respectively) in *C. gattii* cultures under iron deprivation (LIM). UDP-glucose dehydrogenase is involved indirectly in capsule formation, by catalyzing the conversion of UDP-glucose into UDP-glucuronic acid, which is one of the residues of the capsular component glucuronoxylomannan.⁶⁸ Sequence analysis of the GMC-oxidoreductase demonstrated a 95% amino acid identity with protein Smg1 (AAM83033) from *Filobasidiella neoformans* (teleomorph-typified name to *C. neoformans*). Smg1 is suggested to play overlapping roles with signaling components in the regulation of the cAMP-signalling pathways, which in turn regulates mating, capsule formation, and melanin production.^{61, 69} Protein wos2, associated with cell signaling, was also upregulated upon iron deprivation (3.5-fold). These observations may suggest that iron deprivation and virulence-related functions are connected in *C. gattii* R265.

A voltage-dependent ion-selective channel (VDAC) manifested the most expressive fold change in *C. gattii* cultures under iron deprivation (LIM) after cultivation of yeast cells in LIM+Fe (17-fold, in comparison with LIM). Since VDAC is involved with mitochondrial signaling⁷⁰, we speculate that it could participate in iron homeostasis, although this phenomenon has no precedent in the literature.

Our results show a fluctuation in the expression of metabolic proteins when *C. gattii* was cultivated in LIM or LIM+Fe (Table 2 and 3). As expected, proteins involved in mitochondrial pathways such as the TCA cycle (ATP citrate synthase, aconitase), pyruvate metabolism (malate synthase) and oxidative phosphorylation (ubiquinol-cytochrome C reductase, ATP synthase beta subunit, H⁺-transporting two sector ATPase subunit alpha) were upregulated. Proteins with roles in amino acid metabolism (saccharopine dehydrogenase, glycine hydroxymethyltransferase, methionine adenosyltransferase), fatty acid metabolism (acyl-CoA dehydrogenase), and fatty acid biosynthesis (acetyl-CoA carboxylase, fatty acid synthase beta subunit) were overexpressed in *C. gattii* upon iron replete condition of growth. These metabolic response differences of *C. gattii* grown in LIM and LIM+Fe reflect the iron influence on regulation of catabolic and anabolic events, which was also evidenced in *C. neoformans*.³¹

Many proteins related to assembly of ribosome subunits (40S and 60S) were upregulated under iron availability (Table 3). This evidence, also described for *C. neoformans*³¹, suggests that protein biosynthesis is in part modulated by iron in *C. gattii*.

3.6. Validation of differential protein expression analysis

Protease assays were applied to further validate the proteomic data. Figure 3 shows the activity against two 7-AMC substrates; the total protease and specific metalloprotease activities were higher when *C. gattii* was grown in LIM as compared to LIM+Fe.

GAPDH expression was also used as a prototype for experimental validation of differential expression proteomic results. The GAPDH western blot (Figure 4) supports our MudPIT result indicating a greater abundance when *C. gattii* was grown in LIM, as shown by the histogram obtained from the densitometry analysis using the IMAGE J program (2.7-fold, as compared to LIM+Fe).

3.7. A proteomic and transcriptomic comparison for *C. gattii*

Here we describe our observations in comparison with available data about proteins involved in iron homeostasis for *C. neoformans*^{28–31, 43, 71} with our *C. gattii* proteomic data. Firstly, the cell surface reductase proteins, which are present in *C. neoformans*^{72, 73}, were not identified in our proteomic data; therefore we carried out a TTC assay to evaluate the presence of cell surface reductase activity in *C. gattii*. As demonstrated on Supplemental Figure S1 (Supporting Information), *C. gattii* presented a cell surface reductase activity similar to that of *C. neoformans* (H99) grown on LIM and LIM+Fe condition.

As for proteins involved in iron uptake, only the Cfo1 (CNBG_4942 acidic laccase) protein was identified in low abundance in our proteomic results from *C. gattii* grown in LIM and LIM+Fe. Cfo1 is paired with Cft1 and together are related to iron uptake.^{29, 71}

At the iron regulation level, the Cir1 (CNBG_5691 iron regulator) protein was identified in our proteomic data from both LIM and LIM+Fe conditions. Cir1 is a general iron regulator that controls the transcript levels of iron-responsive genes.³⁰

Some proteins were exclusively expressed when *C. gattii* was grown in LIM and we speculate that these proteins might influence iron homeostasis in this fungus. In this context,

two proteins with distinct functions enrolled on endocytic pathway were identified. The RAB small monomeric GTPase (CNBG_3206), which is part of RAB GTPases group, a large family of proteins with a variety of regulatory functions in membrane traffic; and the vacuolar protein sorting-associated protein vps27 (CNBG_5311) that is an endosomal protein required for recycling Golgi-proteins, to produce lumenal membranes and to sort ubiquitinated proteins targeted for degradation.⁷⁴ Three distinct peptidases (carboxypeptidase C - CNBG_1925, ubiquitin carboxyl-terminal hydrolase 12 - CNBG_2384, dipeptidyl-peptidase and tripeptidyl-peptidase - CNBG_5044) were also identified uniquely from *C. gattii* grown in LIM condition. This observation corroborates our results concerning enhanced peptidase activity that *C. gattii* showed when grown in LIM as compared to LIM+Fe. In addition, a general transcriptional repressor protein (CNBG_3041), which has a tetratricopeptide repeat (TPR) motif, was expressed by *C. gattii* only in LIM. Most TPR-containing proteins are associated with multiprotein complexes, and there is extensive evidence indicating that TPR motifs are important to the functioning of chaperones, cell-cycle, transcription, and protein transport complexes.⁷⁵

Altogether, our data suggest that *C. gattii* has some iron homeostasis-related proteins that might be different from the ones previously described in *C. neoformans* according our experimental design.

3.8. Two- dimensional analysis and comparison with MudPIT results

According to comparative image analysis ten spots having differential volumes among classes were selected, excised from gels in both conditions, and identified using LC-MS/MS (Figure 5). A total of 61 proteins were identified. Table 4 shows the proteins identified by 2D-GE and the comparison of this analysis with the MudPIT data; an overlapping of some 90% (40 proteins) was observed when comparing these two methodologies. Five of the proteins found after the combination of 2D-GE and LC-MS/MS were not found by MudPIT.

Although protein separation using 2D-GE is a powerful methodology, a single spot may contain more than one protein as frequently observed in LC-MS/MS analyses.⁷⁶ It is also important to note that of these 61 proteins, 16 of them were found in two or more different spots; this frequently happens due to protein isoforms or post-translational modifications.^{77, 78}

To evaluate if the similarity of the global proteomic profile obtained using 2D-GE and MudPIT, we classified proteins from 2D-GE according to GO terms. Figure 6 shows that the functional profile was similar for both techniques. Nevertheless, 5 proteins were identified exclusively by 2D-GE showing that these methods can yield complementary results.

We also investigate pathways related to mitochondrial functions that were similarly represented using both approaches such as glycolysis/gluconeogenesis (phosphoglycerate kinase, pyruvate dehydrogenase e1 component beta subunit, phosphoenolpyruvate carboxykinase, glyceraldehyde-3-phosphate dehydrogenase and phosphopyruvate hydratase), TCA cycle (succinyl-CoA ligase beta-chain and pyruvate dehydrogenase e1 component beta subunit), pentose phosphate pathway (phosphogluconate dehydrogenase), and oxidative phosphorylation (ATP synthase beta subunit). The succinyl-CoA ligase beta-chain protein (involved in TCA cycle pathway) was identified only in 2D-GE being one of the most represented pathways by the KEGG analysis of our MudPIT data.

4. DISCUSSION

Shotgun proteomic techniques such as MudPIT are considered state-of-the-art tools for studying complex protein mixtures.⁴⁴ MudPIT is able to identify thousands of proteins with

great sensitivity and accuracy by using a combination of strong cation exchange with reverse phase chromatography.⁷⁹ In our study, we applied MudPIT to investigate the proteomic profiling of *C. gattii* grown in low and high iron concentrations. More than 1,000 proteins were identified in both conditions tested; we believe this is the most extensive effort so far to characterize this organism's proteome.

Iron is essential to most living organisms and they have developed sophisticated ways to use this element in their regular metabolisms. For pathogenic microbes, iron is required for the expression of virulence factors and, consequently, for the success of the infectious process.^{80, 81} Many studies evaluating the response of pathogens to iron starvation have been developed.^{80, 82–87} In *C. neoformans*, the metabolic machinery required for iron homeostasis has been studied in detail.^{26–32, 71} Proteins operating in this system include exported reductants, cell surface reductases, a permease/ferroxidase complex for iron uptake, siderophore utilization (iron bound siderophores secreted by other microbes) and iron sensing/regulation proteins.^{34, 28} These elements are determinants of virulence mechanisms in this pathogen. Although the studies mentioned above have been based in different approaches, the comparison of our MudPIT data obtained with *C. gattii* and the *C. neoformans* literature revealed a similar global response after exposure of the fungus to iron starvation or availability. For example, proteins related to metabolic pathways such as TCA cycle, glycolysis/gluconeogenesis, oxidative phosphorylation were upregulated in iron replete condition in both *C. gattii* and *C. neoformans*.³¹ Similar results were observed for proteins required for assembly of ribosomal subunits, which demonstrate that protein biosynthesis is affected under iron deprivation in both species. However, we observed that the majority of proteins described as regulators of iron homeostasis in *C. neoformans* were not identified in our proteomic analysis. This fact may have occurred due low abundance of these proteins, which render protein identification according our experimental design; or, the iron machinery *C. gattii* and *C. neoformans* has some distinctions (Figure 7). Of the iron-related proteins described for *C. neoformans*, we only identified, in low abundance, one iron uptake protein (Cfo1) and the iron regulator Cir1. Cfo1 is a ferroxidase required for the utilization of inorganic iron and transferring, and for full virulence.⁷¹ Cir1 is responsible for the transcriptional response to iron by regulation both iron uptake and the expression of the major cryptococcal virulence factors.³⁰ In a recent study, Silva and colleagues (2011) reported similarities and differences in genes related to iron homeostasis in the genome of *C. neoformans* var. *grubii* and *C. gattii*. For example, they observed that *C. neoformans* has five metalloreductases homologs and three ferroxidases homologs, while *C. gattii* has three metalloreductases homologs and one for ferroxidases. It was demonstrated in both species at least one homolog gene related to high and low-affinity reductase systems, vacuolar and mitochondrial iron uptake, siderophore transport and heme utilization as iron source.

Alterations in iron triggering are related to many cellular disorders, mainly because iron cofactors activate enzymes involved in essential metabolic processes. For example, iron cofactors are involved in DNA repair, xenobiotic metabolism, and biosynthesis of amino acids, proteins, sterols and fatty acids.⁸⁰ Iron-related proteins are required for oxidation of acetyl CoA via the TCA cycle and generation of ATP via oxidative phosphorylation. Synthesis of heme and iron-sulfur clusters need iron to occur.^{88, 89} Although iron homeostasis is clearly essential for cellular metabolism, little is known about the specific molecular mechanisms required to override iron deficiency. Studies in *S. cerevisiae* indicate this yeast responds to iron deficiency by activating iron uptake systems, mobilizing iron stores, optimizing metal use and regulating gene expression at both transcriptional and posttranscriptional levels.⁹⁰ With regards to posttranscriptional mechanisms, under iron starvation, yeast cells activate the expression of proteins that degrade mRNAs encoding proteins involved in iron pathways.^{88, 91} This mechanism has also been described in bacteria.⁹² In our study, two metalloproteases were highly expressed under iron starvation

(Table 2). Evaluation of specific metalloprotease activity of cytoplasmic extract of *C. gattii* grown in LIM and LIM+Fe showed a correlation between activity and protein abundance (Figure 3). Total protease activity was also more accentuated in *C. gattii* after growth in LIM than LIM+Fe and this evidence is in accord with the presence of 3 distinct peptidases identified uniquely from *C. gattii* grown in LIM. These observations led us to hypothesize that a posttranslational regulation may occur in *C. gattii* under iron deprivation, which might utilize proteases to degrade iron-dependent proteins or iron cofactors. However, future experiments, such as an evaluation of the specificity of these enzymes (Figure 7), are required to rigorously prove this hypothesis. We note that our reported metalloprotease activity might not accurately reflect the activity from secreted proteases as our quantitation was not limited to cytoplasmic extracts. Nevertheless, it is reasonable to hypothesize that the abundant metalloproteases we observed in the LIM condition should yield, to some degree, in abundant extracellular metalloprotease. In this regard, we suggest future studies to target the extracellular proteolytic activity from *C. gattii* cells grown under iron-depleted and replete conditions. This might aid in further understanding the involvement of iron deprivation in the establishment of cryptococcosis, similarly as in previous works that have linked extracellular proteolytic activity with a role in the dissemination of cryptococcosis in *C. neoformans*⁹³⁻⁹⁵

Studies in *C. neoformans* demonstrated that iron regulates the expression of classical virulence factors such as production of a polysaccharide capsule, the deposition of melanin at the cell wall, and the ability to grow at 37 °C.^{27, 30, 71} Similar observations were described for bacterial pathogens.^{87, 96, 97} In our study, the UDP-glucose dehydrogenase related to capsule formation was highly expressed after growth of *C. gattii* in LIM. It is a key enzyme involved in the biosynthesis of UDP-glucuronic acid, which is part of the main capsule component called glucuronoxylomannan (GXM). GMC-oxidoreductase, also called Smg1 protein, was also upregulated under these conditions. This protein is likely involved in the regulation of cAMP-signaling pathways. Considering that these pathways are indirectly enrolled in capsule and melanin production in *C. neoformans*^{98, 99}, we speculate that a correlation between iron levels and the expression of virulence factors may exist in *C. gattii*.

The mitochondrion has a crucial role in maintaining cellular iron homeostasis. Ma and colleagues,²³ analyzed 24 *C. gattii* Vancouver Island isolates and proposed that they have a change in mitochondrial regulation, which led to a particular increased capacity to survive and proliferate inside macrophages resulting in a hypervirulent phenotype. As cited above, *C. gattii* R265 was isolated from patients of the Vancouver Island outbreak.⁷ In our study, we observed that proteins involved in TCA cycle, glycolysis/gluconeogenesis and oxidative phosphorylation were more abundantly expressed when iron was available in the culture medium, suggesting that iron depletion affects the mitochondrial function in *C. gattii*. Another mitochondrion-related protein affected by iron availability was VDAC, a gatekeeper for the entry and exit of mitochondrial metabolites.⁷⁰ This molecule was highly expressed in our LIM+Fe condition. The connections of VDAC with iron metabolism are still unknown, but our results suggest that this protein is putatively involved in maintaining iron homeostasis in *C. gattii*. Additional experimentation is required to evaluate if iron levels influence the hypervirulent genotype of the R265 isolate of *C. gattii*.

As well-described previously, MudPIT is a high-throughput methodology for analyses of proteomes showing unquestionable better performance than gel-based analysis. However, this technique is still not as popular as two-dimensional gel electrophoresis, even for analyzing complex mixtures.^{100, 101} Therefore, we performed 2D-GE with same conditions used in MudPIT analysis and we compared the results. The functional profile of proteins identified from 2D-GE gels matched with the profile obtained in MudPIT data. Nevertheless, in our hands, the approaches showed to be somewhat complementary.

To our knowledge, our data provide the first efforts in describing a proteomic view of iron deprivation in *C. gattii*. We suggest future works to use complementary strategies to investigate the expression of unidentified iron-related proteins that can be of key importance for understanding the influence of iron homeostasis in the pathogenesis of *C. gattii*. Transcriptional and metabolic studies will also be required to further validate these data and the role of iron homeostasis in this emerging pathogenic species.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

We thank Prof. Marcio Lourenço Rodrigues for kindly revising the manuscript and for his invaluable suggestions. This work was supported by Brazilian Grants from CNPq, CAPES and MCT/FINEP/Rede GENOPROT (grant number 01.07.0552.00) and by NIH P41-RR011823. PCC and JS GF were funded by CAPES-Fiocruz 30/2006 and PDTIS. JC is a Ph.D. student at Centro de Biotecnologia (Universidade Federal do Rio Grande do Sul, Brazil) and received a scholarship (grant number 201227/2009-6) for a 6 months stay at The Scripps Research Institute, USA.

REFERENCES

1. Morgan J, McCarthy KM, Gould S, Fan K, Arthington-Skaggs B, Iqbal N, Stamey K, Hajjeh RA, Brandt ME. *Cryptococcus gattii* infection: characteristics and epidemiology of cases identified in a South African province with high HIV seroprevalence 2002–2004. *Clin Infect Dis*. 2006; 43:1077–1080. [PubMed: 16983624]
2. Litvintseva AP, Thakur R, Reller LB, Mitchell TG. Prevalence of clinical isolates of *Cryptococcus gattii* serotype C among patients with AIDS in Sub-Saharan Africa. *J Infect Dis*. 2005; 192:888–892. [PubMed: 16088839]
3. Sorrell TC. *Cryptococcus neoformans* variety *gattii*. *Med Mycol*. 2001; 39(2):155–168. [PubMed: 11346263]
4. Chayakulkeeree M, Perfect JR. *Cryptococcosis*. *Infect Dis Clin North Am*. 2006; 20(3):507–544. v–vi. [PubMed: 16984867]
5. Idnurm A, Bahn YS, Nielsen K, Lin X, Fraser JA, Heitman J. Deciphering the model pathogenic fungus *Cryptococcus neoformans*. *Nat Rev Microbiol*. 2005; 3(10):753–764. [PubMed: 16132036]
6. Casadevall, A.; Perfect, JR. *Cryptococcus neoformans*. Washington, D.C: ASM Press; 1998.
7. Kidd SE, Hagen F, Tschärke RL, Huynh M, Bartlett KH, Fyfe M, Macdougall L, Boekhout T, Kwon-Chung KJ, Meyer W. A rare genotype of *Cryptococcus gattii* caused the cryptococcosis outbreak on Vancouver Island (British Columbia, Canada). *Proc Natl Acad Sci U S A*. 2004; 101(49):17258–17263. [PubMed: 15572442]
8. Lester SJ, Malik R, Bartlett KH, Duncan CG. *Cryptococcosis*: update and emergence of *Cryptococcus gattii*. *Vet Clin Pathol*. 2011
9. Galanis E, Macdougall L. Epidemiology of *Cryptococcus gattii*, British Columbia, Canada, 1999–2007. *Emerg Infect Dis*. 2010; 16(2):251–257. [PubMed: 20113555]
10. Nicol AM, Hurrell C, McDowall W, Bartlett K, Elmieh N. Communicating the risks of a new, emerging pathogen: the case of *Cryptococcus gattii*. *Risk Anal*. 2008; 28(2):373–386. [PubMed: 18419655]
11. Duncan C, Stephen C, Campbell J. Clinical characteristics and predictors of mortality for *Cryptococcus gattii* infection in dogs and cats of southwestern British Columbia. *Can Vet J*. 2006; 47(10):993–998. [PubMed: 17078248]
12. Kronstad JW, Attarian R, Cadieux B, Choi J, D'Souza CA, Griffiths EJ, Geddes JM, Hu G, Jung WH, Kretschmer M, Saikia S, Wang J. Expanding fungal pathogenesis: *Cryptococcus* breaks out of the opportunistic box. *Nat Rev Microbiol*. 2011; 9:193–203. [PubMed: 21326274]
13. D'Souza CA, Kronstad JW, Taylor G, Warren R, Yuen M, Hu G, Jung WH, Sham A, Kidd SE, Tangen K, Lee N, Zeilmaker T, Sawkins J, McVicker G, Shah S, Gerner S, Griggs A, Zeng Q, Bartlett K, Li W, Wang X, Heitman J, Stajich JE, Fraser JA, Meyer W, Carter D, Schein J,

- Krzywinski M, Kwon-Chung KJ, Varma A, Wang J, Brunham R, Fyfe M, Ouellette BF, Siddiqui A, Marra M, Jones S, Holt R, Birren BW, Galagan JE, Cuomo CA. Genome Variation in *Cryptococcus gattii*, an Emerging Pathogen of Immunocompetent Hosts. *MBio*. 2011; 2(1):e00342-10.
14. Ngamskulrungronj P, Price J, Sorrell T, Perfect JR, Meyer W. *Cryptococcus gattii* Virulence Composite: Candidate Genes Revealed by Microarray Analysis of High and Less Virulent Vancouver Island Outbreak Strains. *PLoS One*. 2011; 6(1):e16076. [PubMed: 21249145]
 15. Ngamskulrungronj P, Gilgado F, Faganello J, Litvintseva AP, Leal AL, Tsui KM, Mitchell TG, Vainstein MH, Meyer W. Genetic diversity of the *Cryptococcus* species complex suggests that *Cryptococcus gattii* deserves to have varieties. *PLoS One*. 2009; 4(6):e5862. [PubMed: 19517012]
 16. Frealle E, Noel C, Viscogliosi E, Camus D, Dei-Cas E, Delhaes L. Manganese superoxide dismutase in pathogenic fungi: an issue with pathophysiological and phylogenetic involvements. *FEMS Immunol Med Microbiol*. 2005; 45:411–422. [PubMed: 16055318]
 17. Hicks JK, Heitman J. Divergence of protein kinase A catalytic subunits in *Cryptococcus neoformans* and *Cryptococcus gattii* illustrates evolutionary reconfiguration of a signaling cascade. *Eukaryot Cell*. 2007; 6(3):413–420. [PubMed: 17189488]
 18. Latouche GN, Sorrell TC, Meyer W. Isolation and characterisation of the phospholipase B gene of *Cryptococcus neoformans* var. *gattii*. *FEMS Yeast Res*. 2002; 2(4):551–561. [PubMed: 12702271]
 19. Narasipura, SD.; Chaturvedi, V.; Chaturvedi, S. *Mol Microbiol*. Vol. 55. England: 2005. Characterization of *Cryptococcus neoformans* variety *gattii* SOD2 reveals distinct roles of the two superoxide dismutases in fungal biology and virulence; p. 1782-1800.
 20. Ren P, Springer DJ, Behr MJ, Samsonoff WA, Chaturvedi S, Chaturvedi V. Transcription factor STE12alpha has distinct roles in morphogenesis, virulence, and ecological fitness of the primary pathogenic yeast *Cryptococcus gattii*. *Eukaryot Cell*. 2006; 5(7):1065–1080. [PubMed: 16835451]
 21. Wright LC, Payne J, Santangelo RT, Simpanya MF, Chen SC, Widmer F, Sorrell TC. Cryptococcal phospholipases: a novel lysophospholipase discovered in the pathogenic fungus *Cryptococcus gattii*. *Biochem J*. 2004; 384:377–384. [PubMed: 15320865]
 22. Ngamskulrungronj P, Himmelreich U, Breger JA, Wilson C, Chayakulkeeree M, Krockenberger MB, Malik R, Daniel HM, Toffaletti D, Djordjevic JT, Mylonakis E, Meyer W, Perfect JR. The trehalose synthesis pathway is an integral part of the virulence composite for *Cryptococcus gattii*. *Infect Immun*. 2009; 77(10):4584–4596. [PubMed: 19651856]
 23. Ma H, Hagen F, Stekel DJ, Johnston SA, Sionov E, Falk R, Polacheck I, Boekhout T, May RC. The fatal fungal outbreak on Vancouver Island is characterized by enhanced intracellular parasitism driven by mitochondrial regulation. *Proc Natl Acad Sci U S A*. 2009; 106(31):12980–12985. [PubMed: 19651610]
 24. Dimmer KS, Rapaport D. Proteomic view of mitochondrial function. *Genome Biol*. 2008; 9:209. [PubMed: 18331620]
 25. Levi S, Rovida E. The role of iron in mitochondrial function. *Biochim Biophys Acta*. 2009; 1790(7):629–636. [PubMed: 18948172]
 26. Jung WH, Saikia S, Hu G, Wang J, Fung CK, D'Souza C, White R, Kronstad JW. HapX positively and negatively regulates the transcriptional response to iron deprivation in *Cryptococcus neoformans*. *PLoS Pathog*. 2010; 6(11):e1001209.
 27. Jung WH, Kronstad JW. The iron-responsive, GATA-type transcription factor *cir1* influences mating in *Cryptococcus neoformans*. *Mol Cells*. 2010; 31(1):73–77. [PubMed: 21120626]
 28. Jung WH, Kronstad JW. Iron and fungal pathogenesis: a case study with *Cryptococcus neoformans*. *Cell Microbiol*. 2008; 10(2):277–284. [PubMed: 18042257]
 29. Jung WH, Sham A, Lian T, Singh A, Kosman DJ, Kronstad JW. Iron source preference and regulation of iron uptake in *Cryptococcus neoformans*. *PLoS Pathog*. 2008; 4(2):e45. [PubMed: 18282105]
 30. Jung WH, Sham A, White R, Kronstad JW. Iron regulation of the major virulence factors in the AIDS-associated pathogen *Cryptococcus neoformans*. *PLoS Biol*. 2006; 4(12):e410. [PubMed: 17121456]

31. Lian T, Simmer MI, D'Souza CA, Steen BR, Zuyderduyn SD, Jones SJ, Marra MA, Kronstad JW. Iron-regulated transcription and capsule formation in the fungal pathogen *Cryptococcus neoformans*. *Mol Microbiol*. 2005; 55(5):1452–1472. [PubMed: 15720553]
32. Jacobson ES, Goodner AP, Nyhus KJ. Ferrous iron uptake in *Cryptococcus neoformans*. *Infect Immun*. 1998; 66(9):4169–4175. [PubMed: 9712764]
33. Barluzzi R, Saleppico S, Nocentini A, Boelaert JR, Neglia R, Bistoni F, Blasi E. Iron overload exacerbates experimental meningoencephalitis by *Cryptococcus neoformans*. *J Neuroimmunol*. 2002; 132(1–2):140–146. [PubMed: 12417444]
34. Silva M, Schrank A, Bailão EF, Bailão AM, Borges CL, Staats CC, Parente JA, Pereira M, Salem-Izacc SM, Mendes-Giannini MJS, Zancopé-Oliveira RM, Rosa e Silva LK, Nosanchuk JD, Vainstein MH, Soares CM. The homeostasis of iron, copper and zinc in *Paracoccidioides brasiliensis*, *Cryptococcus neoformans* var. *grubii* and *Cryptococcus gattii*: A comparative analysis. *Frontiers in Microbiology*. 2011; 2:1–15. [PubMed: 21716958]
35. Fischer Jde S, Liao L, Carvalho PC, Barbosa VC, Domont GB, Carvalho Mda G, Yates JR 3rd. Dynamic proteomic overview of glioblastoma cells (A172) exposed to perillyl alcohol. *J Proteomics*. 2010; 73:1018–1027. [PubMed: 20083244]
36. Zybaylov B, Mosley AL, Sardi ME, Coleman MK, Florens L, Washburn MP. Statistical analysis of membrane proteome expression changes in *Saccharomyces cerevisiae*. *J Proteome Res*. 2006; 5(9):2339–2347. [PubMed: 16944946]
37. Carrozza MJ, Florens L, Swanson SK, Shia WJ, Anderson S, Yates J, Washburn MP, Workman JL. Stable incorporation of sequence specific repressors Ash1 and Ume6 into the Rpd3L complex. *Biochim Biophys Acta*. 2005; 1731:77–87. [PubMed: 16314178]
38. Graumann J, Dunipace LA, Seol JH, McDonald WH, Yates JR, Wold BJ, Deshaies RJ. Applicability of tandem affinity purification MudPIT to pathway proteomics in yeast. *Mol Cell Proteomics*. 2004; 3(3):226–237. [PubMed: 14660704]
39. Wu CC, MacCoss MJ, Mardones G, Finnigan C, Mogelsvang S, Yates JR, Howell KE. Organellar proteomics reveals Golgi arginine dimethylation. *Mol Biol Cell*. 2004; 15(6):2907–2919. [PubMed: 15047867]
40. Washburn MP, Ulaszek RR, Yates JR. Reproducibility of quantitative proteomic analyses of complex biological mixtures by multidimensional protein identification technology. *Anal Chem*. 2003; 75(19):5054–5061. [PubMed: 14708778]
41. Koller A, Washburn MP, Lange BM, Andon NL, Deciu C, Haynes PA, Hays L, Schieltz D, Ulaszek R, Wei J, Wolters D, Yates JR. Proteomic survey of metabolic pathways in rice. *Proc Natl Acad Sci U S A*. 2002; 99(18):11969–11974. [PubMed: 12163647]
42. Florens L, Washburn MP, Raine JD, Anthony RM, Grainger M, Haynes JD, Moch JK, Muster N, Sacci JB, Tabb DL, Witney AA, Wolters D, Wu Y, Gardner MJ, Holder AA, Sinden RE, Yates JR, Carucci DJ. A proteomic view of the *Plasmodium falciparum* life cycle. *Nature*. 2002; 419(6906):520–526. [PubMed: 12368866]
43. Tangen KL, Jung WH, Sham AP, Lian T, Kronstad JW. The iron- and cAMP-regulated gene *SIT1* influences ferrioxamine B utilization, melanization and cell wall structure in *Cryptococcus neoformans*. *Microbiology*. 2007; 153(Pt 1):29–41. [PubMed: 17185532]
44. Washburn MP, Wolters D, Yates JR. Large-scale analysis of the yeast proteome by multidimensional protein identification technology. *Nat Biotechnol*. 2001; 19(3):242–247. [PubMed: 11231557]
45. McDonald WH, Tabb DL, Sadygov RG, MacCoss MJ, Venable J, Graumann J, Johnson JR, Cociorva D, Yates JR. MS1, MS2, and SQT-three unified, compact, and easily parsed file formats for the storage of shotgun proteomic spectra and identifications. *Rapid Commun Mass Spectrom*. 2004; 18(18):2162–2168. [PubMed: 15317041]
46. Eng J, McCormack A, Yates JR. An approach to correlate tandem mass spectral data of peptides with amino acid sequences in a protein database. *Journal of the American Society for Mass Spectrometry*. 1994; 5(11):976–989.
47. Tabb DL, McDonald WH, Yates JR 3rd. DTASelect and Contrast: tools for assembling and comparing protein identifications from shotgun proteomics. *J Proteome Res*. 2002; 1(1):21–26. [PubMed: 12643522]

48. Carvalho PC, Yates Iii JR, Barbosa VC. Analyzing shotgun proteomic data with PatternLab for proteomics. Chapter 13. Curr Protoc Bioinformatics. 2010 Unit 13.13.1-15.
49. Carvalho PC, Fischer JS, Chen EI, Yates JR 3rd, Barbosa VC. PatternLab for proteomics: a tool for differential shotgun proteomics. BMC Bioinformatics. 2008; 9:316. [PubMed: 18644148]
50. Carvalho PC, Fischer JS, Chen EI, Domont GB, Carvalho MG, Degraive WM, Yates JR, Barbosa VC. GO Explorer: A gene-ontology tool to aid in the interpretation of shotgun proteomics data. Proteome Sci. 2009; 7:6. [PubMed: 19239707]
51. Benjamini Y, Hochberg Y. Controlling the False Discovery Rate: a practical and powerful approach to multiple testing. J R Statist Soc B. 1995; 57:289–300.
52. Gotz S, Garcia-Gomez JM, Terol J, Williams TD, Nagaraj SH, Nueda MJ, Robles M, Talon M, Dopazo J, Conesa A. High-throughput functional annotation and data mining with the Blast2GO suite. Nucleic Acids Res. 2008; 36:3420–3435. [PubMed: 18445632]
53. Conesa A, Gotz S, Garcia-Gomez JM, Terol J, Talon M, Robles M. Blast2GO: a universal tool for annotation, visualization and analysis in functional genomics research. Bioinformatics. 2005; 21:3674–3676. [PubMed: 16081474]
54. Kanehisa M, Goto S. KEGG: kyoto encyclopedia of genes and genomes. Nucleic Acids Res. 2000; 28:27–30. [PubMed: 10592173]
55. Niessen S, Mcleod I, Yates Jr. Silver Staining, Digestion, and Extraction of Peptides from an Acrylamide Gel for MS Analysis. Cold Spring Harbor Protocols. 2006
56. Gatlin CL, Kleemann GR, Hays LG, Link AJ, Yates JR 3rd. Protein identification at the low femtomole level from silver-stained gels using a new fritless electrospray interface for liquid chromatography-microspray and nanospray mass spectrometry. Anal Biochem. 1998; 263:93–101. [PubMed: 9750149]
57. Oliveira MC, Hirata IY, Chagas JR, Boschcov P, Gomes RA, Figueiredo AF, Juliano L. Intramolecularly quenched fluorogenic peptide substrates for human renin. Anal Biochem. 1992; 203:39–46. [PubMed: 1524216]
58. Barbosa MS, Bao SN, Andreotti PF, de Faria FP, Felipe MS, dos Santos Feitosa L, Mendes-Giannini MJ, Soares CM. Glyceraldehyde-3-phosphate dehydrogenase of *Paracoccidioides brasiliensis* is a cell surface protein involved in fungal adhesion to extracellular matrix proteins and interaction with cells. Infect Immun. 2006; 74:382–389. [PubMed: 16368993]
59. Ogur M, St. John R, Nagai S. Tetrazolium overlay technique for population studies of respiration deficiency in yeast. Science. 1957; 125(3254):928–929. [PubMed: 13421693]
60. Ashburner M, Ball CA, Blake JA, Botstein D, Butler H, Cherry JM, Davis AP, Dolinski K, Dwight SS, Eppig JT, Harris MA, Hill DP, Issel-Tarver L, Kasarskis A, Lewis S, Matese JC, Richardson JE, Ringwald M, Rubin GM, Sherlock G. Gene ontology: tool for the unification of biology. The Gene Ontology Consortium. Nat Genet. 2000; 25(1):25–29. [PubMed: 10802651]
61. Maeng S, Ko YJ, Kim GB, Jung KW, Floyd A, Heitman J, Bahn YS. Comparative transcriptome analysis reveals novel roles of the Ras and cyclic AMP signaling pathways in environmental stress response and antifungal drug sensitivity in *Cryptococcus neoformans*. Eukaryot Cell. 2010; 9(3): 360–378. [PubMed: 20097740]
62. Jobbins SE, Hill CJ, D'Souza-Basseal JM, Padula MP, Herbert BR, Krockenberger MB. Immunoproteomic approach to elucidating the pathogenesis of cryptococcosis caused by *Cryptococcus gattii*. J Proteome Res. 2010; 9(8):3832–3841. [PubMed: 20545298]
63. Hwang NR, Yim SH, Kim YM, Jeong J, Song EJ, Lee Y, Lee JH, Choi S, Lee KJ. Oxidative modifications of glyceraldehyde-3-phosphate dehydrogenase play a key role in its multiple cellular functions. Biochem J. 2009; 423:253–264. [PubMed: 19650766]
64. Broetto L, Da Silva WO, Bailao AM, De Almeida Soares C, Vainstein MH, Schrank A. Glyceraldehyde-3-phosphate dehydrogenase of the entomopathogenic fungus *Metarhizium anisopliae*: cell-surface localization and role in host adhesion. FEMS Microbiol Lett. 2010; 312(2): 101–109. [PubMed: 20958787]
65. Dill BD, Dessus-Babus S, Raulston JE. Identification of iron-responsive proteins expressed by *Chlamydia trachomatis* reticulate bodies during intracellular growth. Microbiology. 2009; 155:210–219. [PubMed: 19118361]

66. Latifi A, Jeanjean R, Lemeille S, Havaux M, Zhang CC. Iron starvation leads to oxidative stress in *Anabaena* sp. strain PCC 7120. *J Bacteriol.* 2005; 187(18):6596–6598. [PubMed: 16159797]
67. Zaragoza O, Fries BC, Casadevall A. Induction of capsule growth in *Cryptococcus neoformans* by mammalian serum and CO(2). *Infect Immun.* 2003; 71(11):6155–6164. [PubMed: 14573631]
68. Moyrand F, Janbon G. UGD1, encoding the *Cryptococcus neoformans* UDP-glucose dehydrogenase, is essential for growth at 37 degrees C and for capsule biosynthesis. *Eukaryot Cell.* 2004; 3(6):1601–1608. [PubMed: 15590833]
69. D'Souza CA, Alspaugh JA, Yue C, Harashima T, Cox GM, Perfect JR, Heitman J. Cyclic AMP-dependent protein kinase controls virulence of the fungal pathogen *Cryptococcus neoformans*. *Mol Cell Biol.* 2001; 21(9):3179–3191. [PubMed: 11287622]
70. Shoshan-Barmatz V, De Pinto V, Zweckstetter M, Raviv Z, Keinan N, Arbel N. VDAC, a multi-functional mitochondrial protein regulating cell life and death. *Mol Aspects Med.* 2010; 31:227–285. [PubMed: 20346371]
71. Jung WH, Hu G, Kuo W, Kronstad JW. Role of ferroxidases in iron uptake and virulence of *Cryptococcus neoformans*. *Eukaryot Cell.* 2009; 8(10):1511–1520. [PubMed: 19700638]
72. Nyhus KJ, Wilborn AT, Jacobson ES. Ferric iron reduction by *Cryptococcus neoformans*. *Infect Immun.* 1997; 65(2):434–438. [PubMed: 9009293]
73. Jacobson ES, Hong JD. Redox buffering by melanin and Fe(II) in *Cryptococcus neoformans*. *J Bacteriol.* 1997; 179(17):5340–5346. [PubMed: 9286986]
74. Leung K, Dacks J, Field M. Evolution of the Multivesicular Body ESCRT Machinery; Retention across the Eukaryotic lineage. *Traffic.* 2008; 9:1–18. [PubMed: 17908284]
75. Blatch G, Lässle M. The tetratricopeptide repeat: a structural motif mediating protein-protein interactions. *Bioessays.* 1999; 21:932–939. [PubMed: 10517866]
76. Lim H, Eng J, Yates JR 3rd, Tollaksen SL, Giometti CS, Holden JF, Adams MW, Reich CI, Olsen GJ, Hays LG. Identification of 2D-gel proteins: a comparison of MALDI/TOF peptide mass mapping to mu LC-ESI tandem mass spectrometry. *J Am Soc Mass Spectrom.* 2003; 14:957–970. [PubMed: 12954164]
77. Ambatipudi K, Old J, Guilhaus M, Raftery M, Hinds L, Deane E. Proteomic analysis of the neutrophil proteins of the tammar wallaby (*Macropus eugenii*). *Comp Biochem Physiol Part D Genomics Proteomics.* 2006; 1:283–291. [PubMed: 20483260]
78. Berkow RL, Dodson RW. Tyrosine-specific protein phosphorylation during activation of human neutrophils. *Blood.* 1990; 75(12):2445–2452. [PubMed: 1693534]
79. Yates JR 3rd. Mass spectral analysis in proteomics. *Annu Rev Biophys Biomol Struct.* 2004; 33:297–316. [PubMed: 15139815]
80. Sutak R, Lesuisse E, Tachezy J, Richardson DR. Crusade for iron: iron uptake in unicellular eukaryotes and its significance for virulence. *Trends Microbiol.* 2008; 16(6):261–268. [PubMed: 18467097]
81. Weinberg ED. Suppression of bacterial biofilm formation by iron limitation. *Med Hypotheses.* 2004; 63(5):863–865. [PubMed: 15488661]
82. Pieper R, Huang ST, Parmar PP, Clark DJ, Alami H, Fleischmann RD, Perry RD, Peterson SN. Proteomic analysis of iron acquisition, metabolic and regulatory responses of *Yersinia pestis* to iron starvation. *BMC Microbiol.* 2010; 10:30. [PubMed: 20113483]
83. Vidakovics ML, Paba J, Lamberti Y, Ricart CA, de Sousa MV, Rodriguez ME. Profiling the *Bordetella pertussis* proteome during iron starvation. *J Proteome Res.* 2007; 6(7):2518–2528. [PubMed: 17523612]
84. Knight SA, Vilaire G, Lesuisse E, Dancis A. Iron acquisition from transferrin by *Candida albicans* depends on the reductive pathway. *Infect Immun.* 2005; 73(9):5482–5492. [PubMed: 16113264]
85. Cherayil BJ. The role of iron in the immune response to bacterial infection. *Immunol Res.* 2010
86. Ramanan N, Wang Y. A high-affinity iron permease essential for *Candida albicans* virulence. *Science.* 2000; 288(5468):1062–1064. [PubMed: 10807578]
87. Ratledge C. Iron metabolism and infection. *Food Nutr Bull.* 2007; 28(4 Suppl):S515–S523. [PubMed: 18297890]

88. Shakoury-Elizeh M, Protchenko O, Berger A, Cox J, Gable K, Dunn TM, Prinz WA, Bard M, Philpott CC. Metabolic response to iron deficiency in *Saccharomyces cerevisiae*. *J Biol Chem*. 2010; 285(19):14823–14833. [PubMed: 20231268]
89. Ozer A, Bruick RK. Non-heme dioxygenases: cellular sensors and regulators jelly rolled into one? *Nat Chem Biol*. 2007; 3:144–153. [PubMed: 17301803]
90. Philpott CC, Protchenko O. Response to iron deprivation in *Saccharomyces cerevisiae*. *Eukaryot Cell*. 2008; 7(1):20–27. [PubMed: 17993568]
91. Puig S, Vergara SV, Thiele DJ. Cooperation of two mRNA-binding proteins drives metabolic adaptation to iron deficiency. *Cell Metab*. 2008; 7:555–564. [PubMed: 18522836]
92. Masse E, Salvail H, Desnoyers G, Arguin M. Small RNAs controlling iron metabolism. *Curr Opin Microbiol*. 2007; 10:140–145. [PubMed: 17383226]
93. Eigenheer RA, Jin Lee Y, Blumwald E, Phinney BS, Gelli A. Extracellular glycosylphosphatidylinositol-anchored mannoproteins and proteases of *Cryptococcus neoformans*. *FEMS Yeast Res*. 2007; 7(4):499–510. [PubMed: 17233760]
94. Rodrigues ML, dos Reis FC, Puccia R, Travassos LR, Alviano CS. Cleavage of human fibronectin and other basement membrane-associated proteins by a *Cryptococcus neoformans* serine proteinase. *Microb Pathog*. 2003; 34(2):65–71. [PubMed: 12623274]
95. Yoo Ji J, Lee YS, Song CY, Kim BS. Purification and characterization of a 43-kilodalton extracellular serine proteinase from *Cryptococcus neoformans*. *J Clin Microbiol*. 2004; 42(2):722–726. [PubMed: 14766843]
96. Radtke A, O’Riordan M. Intracellular innate resistance to bacterial pathogens. *Cell Microbiol*. 2006; 8:1720–1729. [PubMed: 16939532]
97. Ratledge C, Dover LG. Iron metabolism in pathogenic bacteria. *Annu Rev Microbiol*. 2000; 54:881–941. [PubMed: 11018148]
98. Pukkila-Worley R, Gerrald QD, Kraus PR, Boily MJ, Davis MJ, Giles SS, Cox GM, Heitman J, Alspaugh JA. Transcriptional network of multiple capsule and melanin genes governed by the *Cryptococcus neoformans* cyclic AMP cascade. *Eukaryot Cell*. 2005; 4(1):190–201. [PubMed: 15643074]
99. Alspaugh JA, Pukkila-Worley R, Harashima T, Cavallo LM, Funnell D, Cox GM, Perfect JR, Kronstad JW, Heitman J. Adenyl cyclase functions downstream of the G α protein Gpa1 and controls mating and pathogenicity of *Cryptococcus neoformans*. *Eukaryot Cell*. 2002; 1(1):75–84. [PubMed: 12455973]
100. Kline KG, Wu CC. MudPIT analysis: application to human heart tissue. *Methods Mol Biol*. 2009; 528:281–293. [PubMed: 19153700]
101. Gygi SP, Corthals GL, Zhang Y, Rochon Y, Aebersold R. Evaluation of two-dimensional gel electrophoresis-based proteome analysis technology. *Proc Natl Acad Sci U S A*. 2000; 97(17):9390–9395. [PubMed: 10920198]

Figure 1. Distribution of proteins of *C. gattii* obtained after growth of the fungus in LIM and LIM+Fe. Figures A and B represent the distribution and overlap between the biological replicates from LIM (A) and LIM+Fe (B). Figures C and D show the dispersion of total proteins identified in both conditions, including proteins identified in at least 1 replicate of each condition and proteins present in a minimum of 3 replicates, respectively.

Figure 2. Gene ontology annotation. Molecular functions are represented at multilevel, using a cutoff of 10 sequences, for proteins obtained from LIM and LIM+Fe conditions.

Figure 3.

Peptidase activity of protein extracts of *C. gattii* cells grown under conditions of iron deprivation (LIM) or iron availability (LIM+ Fe) against two different fluorogenic substrates. Substrates *N-t-Boc-Gly-Arg-Arg-MCA* (A) and *N-t-Boc-Ile-Glu-Gly-Arg-MCA* (B) were used in this analysis.

Figure 4.

C. gattii cells GAPDH expression under conditions of iron deprivation (LIM) or iron availability (LIM+ Fe), determined by western blotting. Panel A shows SDS-PAGE stained by Coomassie blue, Panel B, the reactivity against GAPDH heterologous *Paracoccidioides brasiliensis* antibody, and Panel C, a histogram obtained from densitometry analysis of the GAPDH using the IMAGE J program. The experiment was done in triplicate.

Figure 5.
Two-dimensional gels from *C. gattii* grown in iron-depleted (A) and iron replete conditions (B). Arrows indicate spots submitted to LC-MS/MS identification.

Figure 6. Comparison of functional proteomic profiles obtained using MudPIT and 2D-GE techniques. Proteins were annotated according with biological processes and molecular functions terms (level 2) using Blast2GO tool.

Figure 7.

Schematic representation of iron homeostasis in *C. gattii*. Cfo1 and Cir1 are involved in iron homeostasis and virulence in *C. neoformans* and they were identified in this study in *C. gattii*. Many proteins related to iron in *C. neoformans* were detected in *C. gattii* genome. Protease activities were more detected under iron deprivation in *C. gattii* and may be involved in post-translational regulation to maintain iron homeostasis. Figure was updated from Jung & Kronstad 2008.²⁸

Table 1

Putative classification of *C. gattii* hypothetical proteins.

BROAD ID	NCBI ID	NCBI Sequence description	Related species	e-Value	Similarity(%)
Catalytic activity					
CNMG_5689	XP_567571	glutamate synthase (NADH)	<i>C. neoformans</i> var. <i>neoformans</i> JEC21	0	96
CNMG_1228	XP_566910	purine nucleotide biosynthesis-related protein	<i>C. neoformans</i> var. <i>neoformans</i> JEC21	0	99
CNMG_2961	XP_567751	cytochrome c oxidase subunit V	<i>C. neoformans</i> var. <i>neoformans</i> JEC21	7.21919E-48	95
CNMG_5233	XP_571137	isoprenoid biosynthesis-related protein	<i>C. neoformans</i> var. <i>neoformans</i> JEC21	0	98
CNMG_5871	AAN75183	LPD1	<i>C. neoformans</i> var. <i>grubii</i>	0	99
CNMG_5891	XP_570096	histone deacetylation-related protein	<i>C. neoformans</i> var. <i>neoformans</i> JEC21	0	76
Binding					
CNMG_1506	XP_566725	actin filament organization-related protein	<i>C. neoformans</i> var. <i>neoformans</i> JEC21	3.41261E-59	95
CNMG_2009	XP_571615	nonhistone protein 6	<i>C. neoformans</i> var. <i>neoformans</i> JEC21	3.93639E-28	86
CNMG_2725	XP_572710	SCP160 protein	<i>C. neoformans</i> var. <i>neoformans</i> JEC21	0	97
CNMG_2986	XP_567894	protein binding protein	<i>C. neoformans</i> var. <i>neoformans</i> JEC21	3.9348E-123	94
CNMG_3210	XP_567624	endocytosis-related protein	<i>C. neoformans</i> var. <i>neoformans</i> JEC21	0	85
CNMG_5866	Q5y223	electron transfer flavoprotein subunit alpha, mitochondrial	<i>C. gattii</i>	2.1543E-158	98
CNMG_4946	XP_568254	translational initiation-related protein	<i>C. neoformans</i> var. <i>neoformans</i> JEC21	0	97
CNMG_5765	XP_567951	14-3-3 protein	<i>C. neoformans</i> var. <i>neoformans</i> JEC21	5.1547E-136	100
CNMG_0239	XP_566757	heat shock protein	<i>C. neoformans</i> var. <i>neoformans</i> JEC21	0	99
CNMG_3266	XP_569509	heat shock protein 70	<i>C. neoformans</i> var. <i>neoformans</i> JEC21	0	99
CNMG_4499	XP_567978	heat shock protein	<i>C. neoformans</i> var. <i>neoformans</i> JEC21	0	99
CNMG_4857	AAN76524	heat shock protein 90	<i>C. gattii</i>	0	99
CNMG_4912	XP_568283	heat-shock protein	<i>C. neoformans</i> var. <i>neoformans</i> JEC21	0	96
CNMG_5017	XP_568652	heat shock protein	<i>C. neoformans</i> var. <i>neoformans</i> JEC21	1.7379E-117	98
CNMG_5573	XP_569211	heat shock protein	<i>C. neoformans</i> var. <i>neoformans</i> JEC21	0	99
CNMG_5853	AAS92524	HSP12	<i>C. gattii</i>	7.09732E-30	95
CNMG_5963	XP_570059	heat shock protein HSP60	<i>C. neoformans</i> var. <i>neoformans</i> JEC21	0	98
Structural constituent of ribosome					
CNMG_3655	XP_569366	PRCDNA38	<i>C. neoformans</i> var. <i>neoformans</i> JEC21	1.69873E-40	98
CNMG_1225	XP_566913	PRCDNA87	<i>C. neoformans</i> var. <i>neoformans</i> JEC21	1.67055E-66	100
CNMG_2105	XP_571814	structural constituent of ribosome	<i>C. neoformans</i> var. <i>neoformans</i> JEC21	4.53609E-41	99

BROAD ID	NCBI ID	NCBI Sequence description	Related species	e-Value	Similarity(%)
CNBG_5635	XP_567521	ribosomal protein	<i>C. neoformans</i> var. <i>neoformans</i> JEC21	1.27402E-63	99
<i>Miscellaneous</i>					
CNBG_3046	XP_567711	nucleus protein	<i>C. neoformans</i> var. <i>neoformans</i> JEC21	0	96
CNBG_0575	XP_570348	meiotic recombination-related protein	<i>C. neoformans</i> var. <i>neoformans</i> JEC21	0	97
CNBG_5197	XP_571006	mRNA polyadenylation-related protein	<i>C. neoformans</i> var. <i>neoformans</i> JEC21	0	95

Table 2

Upregulated proteins after growth of *C. gattii* in LIM, in comparison to cultivation of the fungus in LIM+Fe. Proteins listed in this table were found to be statistically differentially expressed ($p < 0.01$) and have an absolute fold change greater than 2.5. The set of proteins also satisfies the BH-FDR of 0.05 criterion

[BROAD Institute ID Protein name	Fold change*
Catalytic activity	
<i>hydrolase activity</i>	
[CNBG_2780 peptidase (403 aa)	7.2
[CNBG_6001 extracellular elastinolytic metalloproteinase (814 aa)	6.7
[CNBG_0785 6-phosphogluconolactonase (295 aa)	5.6
[CNBG_3720 beta-1,4-glucosidase (422 aa)	3.1
<i>oxidoreductase activity</i>	
[CNBG_5787 glucose-methanol-choline oxidoreductase (589 aa)	6.9
[CNBG_1866 glyceraldehyde-3-phosphate dehydrogenase (337 aa)	2.7
[CNBG_5326 D-lactaldehyde dehydrogenase (347 aa)	3.8
[CNBG_4706 UDP-glucose dehydrogenase (447 aa)	2.9
[CNBG_6205 3-hydroxyacyl-CoA-dehydrogenase (293 aa)	3.6
Transcription regulator activity	
[CNBG_3163 wos2 protein (226 aa)	3.5
Hypothetical	
[CNBG_3266 conserved hypothetical protein (641 aa)	2.6
[CNBG_0780 conserved hypothetical protein (306 aa)	4.6

* Based on spectral count numbers obtained from LIM and LIM+Fe.

Table 3

Upregulated proteins after growth of *C. gattii* in LIM+Fe, in comparison to cultivation of the fungus in LIM. Proteins listed here were found to be statistically differentially expressed ($p < 0.01$) and have an absolute fold change greater than 2.5. The set of proteins also satisfies the BH-FDR of 0.05 criterion.

[BROAD Institute ID] Protein name	Fold Change[*]
Catalytic activity	
<i>hydrolase activity</i>	
[CNBG_1511] 1,4-alpha-glucan branching enzyme (683 aa)	2.9
<i>ligase activity</i>	
[CNBG_1784] acetyl-CoA carboxylase (2045 aa)	2.8
<i>lyase activity</i>	
[CNBG_0705] aconitase (781 aa)	6.6
<i>oxidoreductase activity</i>	
[CNBG_0069] acyl-CoA dehydrogenase (521 aa)	6.4
[CNBG_5247] fatty acid synthase beta subunit (2515 aa)	5.1
[CNBG_4517] ubiquinol-cytochrome C reductase complex core protein 2 (435 aa)	4.1
[CNBG_2079] mannitol-1-phosphate dehydrogenase (421 aa)	3.6
[CNBG_5912] saccharopine dehydrogenase (396 aa)	2.7
<i>transferase activity</i>	
[CNBG_1012] ATP-citrate synthase (1151 aa)	4.4
[CNBG_1047] glycine hydroxymethyltransferase (500 aa)	3.5
[CNBG_1488] methionine adenosyltransferase (398 aa)	3.7
[CNBG_6151] malate synthase (539 aa)	3.2
Binding	
<i>ion binding</i>	
[CNBG_0737] centromere/microtubule-binding protein cbf5 (504 aa)	3.7
[CNBG_4198] calnexin (555 aa)	4.1
<i>nucleotide binding</i>	
[CNBG_5683] MMS2 protein (811 aa)	4.1
Transporter activity	
[CNBG1793] H ⁺ -transporting two-sector ATPase subunit alpha (541 aa)	7.9
<i>substrate-specific transporter activity</i>	
[CNBG_1632] ATP synthase beta subunit (548 aa)	3.5
<i>transmembrane transporter activity</i>	
[CNBG_3615] voltage-dependent-ion-selective channel (293 aa)	17.0
Transcriptional regulator activity	
[CNBG_4420] nascent polypeptide-associated complex subunit beta (176 aa)	2.8
Structural component of ribosome	
[CNBG_2316] 60S ribosomal protein L24 (151 aa)	3.6
[CNBG_2923] 40S ribosomal protein S0 (293 aa)	2.8
[CNBG_1273] 60S ribosomal protein L7 (251 aa)	2.6
[CNBG_0793] 60S ribosomal protein L18-A (191 aa)	5.7

BROAD Institute ID Protein name	Fold Change [*]
CNBG_0179 60S ribosomal protein S16 (141 aa)	3.2
CNBG_2051 L10e protein (313 aa)	6.6
Hypothetical	
CNBG_1979 conserved hypothetical protein (188 aa)	6.2
CNBG_3648 conserved hypothetical protein (112 aa)	8.0

^{*} Based on Spectral count numbers obtained from LIM and LIM+Fe.

Table 4

Spots from two-dimensional gels (2D-GE) identified by LC-MS/MS.

Spot number	Protein ID description (BROAD database)	Sequence Count LIM/LIM+Fe	Spectral Count LIM/LIM+Fe	Sequence Coverage (%) LIM/LIM+Fe	Sequence Length	MW Exp/Theor.	pI Exp/Theor.	MudPIT analysis
1	CNBG_5018 luminal binding protein	2/-	83/-	4.9/-	371	~32000/40693	~4.4/4.7	+
1	CNBG_4794 DNA polymerase processivity factor	-/5	-/32	-/16.6	344	~32000/38440	~4.4/4.7	+
2	CNBG_1896 formate dehydrogenase	8/7	16/7	25.7/22.3	373	~45000/41200	~4.5/7.3	+
2	CNBG_1355 endopeptidase	10/11	250/273	20.1/18.1	432	~45000/47164	~4.5/4.9	+
2	CNBG_3287 conserved hypothetical protein	3/-	8/-	6.5/-	584	~45000/63175	~4.5/5.3	+
2	CNBG_3266 conserved hypothetical protein	-/5	-/22	-/10.9	640	~45000/69393	~4.5/5.1	+
3	CNBG_1355 endopeptidase	15/7	105/48	30.1/13.9	432	~45000/47164	~4.6/4.9	+
3	CNBG_3687 protein phosphatase type 2C	9/2	12/5	13.2/4.8	516	~45000/54570	~4.6/4.5	+ only in LIM+Fe
3	CNBG_1896 formate dehydrogenase	3/3	3/3	10.7/9.1	373	~45000/41200	~4.6/7.3	+
3	CNBG_3266 conserved hypothetical protein	4/4	4/5	8.1/9.7	640	~45000/69393	~4.6/5.1	+
3	CNBG_4764 aryl-alcohol dehydrogenase	2/2	2/6	5.4/8.4	392	~45000/44111	~4.6/6.5	+
3	CNBG_5573 conserved hypothetical protein	-/4	-/5	-/11	581	~45000/61414	~4.6/5.5	+
3	CNBG_3166 phosphogluconate dehydrogenase	-/2	-/2	-/6.5	491	~45000/53783	~4.6/6.4	+
4	CNBG_4625 pyruvate dehydrogenase e1 component beta subunit	-/2	-/2	-/6.9	407	~40000/43889	~4.8/6.5	+
4	CNBG_3287 conserved hypothetical protein	-/2	-/2	-/4.6	584	~40000/63175	~4.8/5.3	+
4	CNBG_1093 predicted protein	-/2	-/2	-/2.6	1020	~40000/107576	~4.8/4.8	+
5	CNBG_5573 conserved hypothetical protein	38/21	473/164	58.2/31.5	581	~60000/61414	~5.2/5.5	+
5	CNBG_2948 N-terminal asparagine amidohydrolase	2/-	19/-	25.9/-	108	~60000/11560	~5.2/4.5	-
5	CNBG_4706 UDP-glucose dehydrogenase	8/-	24/-	19.3/-	446	~60000/49317	~5.2/5.9	+
5	CNBG_3266 conserved hypothetical protein	8/-	13/-	14.5/-	640	~60000/69393	~5.2/5.1	+
5	CNBG_5787 glucose-methanol-choline oxidoreductase	6/2	10/3	13.3/7.3	588	~60000/65310	~5.2/6	+
5	CNBG_0239 conserved hypothetical protein	5/2	5/3	11.1/6.2	614	~60000/67116	~5.2/5.5	+
5	CNBG_4912 conserved hypothetical protein	3/2	3/4	5.7/5.7	756	~60000/83654	~5.2/5.2	+
5	CNBG_2828 phosphoenolpyruvate carboxykinase	2/-	2/-	4.7/-	551	~60000/61775	~5.2/6.4	+
5	CNBG_1093 predicted protein	2/-	2/-	2.6/-	1020	~60000/107576	~5.2/4.8	+

Spot number	Protein ID description (BROAD database)	Sequence Count LIM/LIM+Fe	Spectral Count LIM/LIM+Fe	Sequence Coverage (%) LIM/LIM+Fe	Sequence Length	MW Exp/Theor.	pI Exp/Theor.	MudPIT analysis
6	[CNBG_5029] acyl-CoA oxidase	22/12	113/67	52.2/28.8	427	~45000/47035	~5.4/5.7	+
6	[CNBG_1346] cAMP-dependent protein kinase inhibitor	3/-	20/-	10.6/-	482	~45000/52354	~5.4/5.2	+
6	[CNBG_1185] succinyl-CoA ligase beta-chain	3/-	15/-	8.4/-	418	~45000/44897	~5.4/6.1	-
6	[CNBG_3266] conserved hypothetical protein	3/-	4/-	6.1/-	640	~45000/69393	~5.4/5.1	+
6	[CNBG_1816] ketol-acid reductoisomerase	3/-	8/-	5/-	401	~45000/44402	~5.4/8	+
6	[CNBG_4857] conserved hypothetical protein	2/-	4/-	3.9/-	699	~45000/79302	~5.4/5	+
6	[CNBG_1632] ATP synthase beta subunit	2/-	2/-	3.7/-	547	~45000/58616	~5.4/6.3	+
6	[CNBG_1618] peptidyl-prolyl cis-trans isomerase D	-/8	-/12	-/26.4	375	~45000/41037	~5.4/6.4	+
7	[CNBG_1632] ATP synthase beta subunit	18/-	158/-	43.7/-	547	~52000/58616	~5.3/6.3	+
7	[CNBG_3197] conserved hypothetical protein	5/-	12/-	14.3/-	510	~52000/54706	~5.3/6.3	+
7	[CNBG_3703] phosphopyruvate hydratase	3/5	4/9	14.1/20.6	433	~52000/46369	~5.3/5.5	+
7	[CNBG_2108] mitochondrial processing peptidase beta subunit	4/-	21/-	10.7/-	478	~52000/52500	~5.3/6	+
7	[CNBG_5573] conserved hypothetical protein	3/-	4/-	7.7/-	581	~52000/61414	~5.3/5.5	+
7	[CNBG_0239] conserved hypothetical protein	2/2	2/2	4.2/7	614	~52000/67116	~5.3/5.5	+
7	[CNBG_4857] conserved hypothetical protein	2/-	4/-	3.1/-	699	~52000/79302	~5.3/5	+
7	[CNBG_0779] 1-phosphatidylinositol-3-phosphate 5-kinase	2/-	2/-	1/-	1997	~52000/220602	~5.3/6	-
8	[CNBG_3703] phosphopyruvate hydratase	21/9	384/140	45.3/283.4	433	~50000/46369	~5.7/5.5	+
8	[CNBG_3166] phosphogluconate dehydrogenase	7/4	9/4	18.3/14.5	491	~50000/53783	~5.7/6.4	+
8	[CNBG_2038] RAB GDP-dissociation inhibitor	4/4	7/4	11.7/12.6	452	~50000/50384	~5.7/5.6	+
8	[CNBG_4857] conserved hypothetical protein	6/-	16/-	8.6/-	699	~50000/79302	~5.7/5	+
8	[CNBG_4912] conserved hypothetical protein	5/-	9/-	8.3/-	756	~50000/83654	~5.7/5.2	+
8	[CNBG_5562] endopeptidase	2/2	6/3	6.7/1	436	~50000/49237	~5.7/5.6	+
8	[CNBG_5240] nucleosome assembly protein 1	2/-	2/-	5.8/-	416	~50000/46872	~5.7/4.4	+
8	[CNBG_1876] conserved hypothetical protein	2/-	14/-	5.1/-	670	~50000/73462	~5.7/6.3	+
8	[CNBG_4834] translation elongation factor EF1-alpha	2/-	4/-	4.1/-	459	~50000/50303	~5.7/9	+
9	[CNBG_3648] conserved hypothetical protein	4/-	147/-	28.8/-	111	~12000/12282	~6.2/6.1	+
9	[CNBG_3023] ubiquitin protein 1	2/-	5/-	6.6/-	381	~12000/42776	~6.2/7.6	-
10	[CNBG_2079] mannitol-1-phosphate dehydrogenase	24/12	223/68	61.2/41.9	420	~38000/44521	~6.8/8.5	+

Spot number	Protein ID description (BROAD database)	Sequence Count LIM/LIM+Fe	Spectral Count LIM/LIM+Fe	Sequence Coverage (%) LIM/LIM+Fe	Sequence Length	MW Exp/Theor.	pI Exp/Theor.	MudPIT analysis
10	[CNBG_0022] conserved hypothetical protein	13/3	16/4	55.6/19.5	338	~38000/36345	~6.8/7.1	+
10	[CNBG_1866] glyceraldehyde-3-phosphate dehydrogenase	10/2	106/6	35.7/10.4	336	~38000/35907	~6.8/7.1	+
10	[CNBG_4654] cysteine synthase	4/-	9/-	15.7/-	363	~38000/38809	~6.8/7.9	+
10	[CNBG_0601] ATP phosphoribosyltransferase	3/-	11/-	14.8/-	357	~38000/37396	~6.8/6.3	+ only on LIM
10	[CNBG_4593] cytoplasm protein	2/-	3/-	11.3/-	346	~38000/37147	~6.8/6.9	+
10	[CNBG_4188] mannitol-1-phosphate dehydrogenase	4/3	28/37	10.8/6.9	361	~38000/37685	~6.8/7.2	+
10	[CNBG_2246] phosphoglycerate kinase	2/-	3/-	7/-	417	~38000/44837	~6.8/6.7	+
10	[CNBG_0705] aconitase	2/-	2/-	4.1/-	780	~38000/84864	~6.8/6.9	+