Published in final edited form as:

Metallomics. 2013 April ; 5(4): 363–371. doi:10.1039/c3mt20220h.

A Copper Hyperaccumulation Phenotype Correlates with Pathogenesis in *Cryptococcus neoformans*

Meera R. Raja¹, Scott R. Waterman², Jin Qiu², Reiner Bleher¹, Peter R. Williamson^{2,3}, and Thomas V. O'Halloran^{1,*}

¹Department of Chemistry, Chemistry of Life Processes Institute, Northwestern University, Evanston, IL, USA

²Laboratory of Clinical Infectious Diseases, National Institute of Allergy and Infectious Diseases, National Institutes of Health, Bethesda, MD, USA

³Section of Infectious Diseases, Department of Medicine, University of Illinois at Chicago, Chicago, IL, USA

INTRODUCTION

Cryptococcus neoformans is a basidiomycetous pathogenic yeast that has emerged as one of the major causative agents of meningoencephalitis in immunocompromised patients, specifically AIDS patients. In sub-Saharan Africa, *Cryptococcus*-mediated meningitis results in approximately 504,000 deaths a year with 70% of the cases having a three month fatality rate. With these numbers, cryptococcal meningitis has risen to become the fourth major cause of death in this region, replacing tuberculosis.¹ This pathogen is also an enduring problem in the developed world, affecting other immunocompromised populations such as solid organ transplant recipients and chemotherapy patients.^{2,34} *Cryptococcus neoformans* possesses a number of mechanisms for survival, and many of them allow it to rapidly adapt and combat the host response that the organism encounters after colonization.^{5,6}

The ability of this fungus to persist in a human host is an important factor in its pathogenesis. Prolonged survival of the pathogen can be dependent on the ability to compete with and acquire essential nutrients from the host organism. For example, all organisms must accumulate transition metals to maintain basal physiological processes and in the host-parasite relationship, acquisition of copper, iron and zinc for these essential cellular processes is highly contested between the two adversaries.^{7,8} Many decades of research have shown an extensive and key role for iron in this relationship across a range of prokaryotic and eukaryotic pathogens;^{9,10,11,12} however, recently an equally combative and informative role for copper is emerging in studies of *Cryptococcus neoformans*.^{13,14,15,16}

Copper is integral to a large number of copper-dependent metalloenzymes,¹⁷ and consequently most cells must accumulate a minimal number of copper atoms so that these metalloproteins can function properly.^{18,19} This basal level of copper that is present in a cell grown under metal limitation conditions, also known as the minimal copper quota, is known for a handful of organisms.^{20,21} The redox chemistry of copper is required as a cofactor in a number of oxygen and antioxidant processing enzymes. However, while it can accept electrons from harmful oxidative sources, excess copper can also be toxic as it can catalyze

Address correspondence to: Thomas V. O'Halloran, Department of Chemistry, Chemistry of Life Processes Institute, Northwestern University, Evanston, IL 60208. Phone: 847-491-5060; Fax: 847-467-4566; t-ohalloran@northwestern.edu.

adventitious radical formation, which can cause damage to cell membranes, nucleic acids, and proteins.^{17,22,23} This duality leads to a tightly regulated transfer system of proteins that efficiently delivers copper to where it is required, but also traps the excess before it becomes detrimental to the cell.^{24,25,26} Copper is of particular importance in the study of *C. neoformans*, as at least two of its copper dependant enzymes, a Cu/Zn SOD1 and a cuprolaccase, are important for the virulence of the pathogen in immunocompromised hosts.^{27,26,27} and are involved in the cell's oxidant defense system.^{28,29}

Expression of proteins involved in copper transport, trafficking, and metabolism is regulated in a copper-responsive manner at the level of transcription in several fungi.^{30,24} The Cuf1 protein is a copper-dependent transcription factor that plays an integral role in the acquisition of the metal by *C. neoformans* by regulating the expression of the high affinity copper transporter Ctr4.¹⁵ Recently, the Cuf1/Ctr4 regulatory system has been demonstrated to be integral to copper acquisition and pathogenesis of *C. neoformans* and has also been shown to be involved in copper detoxification.^{13,31}

The metal environment that *C. neoformans* faces within the host is unclear and may be variable throughout the course of infection. Deletion of the *CUF1* gene not only impairs growth under low copper conditions, this deletion mutant was also significantly less virulent than the wild-type in mammalian brain infection.¹⁴ The protective role of *CUF1* during times of elevated copper is consistent with idea that the pathogen may encounter elevated, potentially toxic levels of the metal during infection.¹³ Using a wild-type strain carrying a CTR4::GFP promoter fusion, it was demonstrated that this fungal pathogen behaves as if it were in a copper-deficient environment during infection of the mouse brain.¹⁵ Previous studies did not observe this copper deficiency in the lung, but recent experiments demonstrate similar expression in both the alveolar macrophages *ex vivo*, as well as copper-chelated *in vitro* environments.¹⁶ This pattern of responses to copper availability during infection, along with the importance of copper-dependent virulence factors of *C. neoformans*,^{32,13} suggests that host and pathogen mechanisms may compete for this essential transition metal. Given the complexity of interplay between the various homeostatic mechanisms in both fungus and host cells, genetic approaches alone are not sufficient.

While many studies have suggested that copper regulation is important to the pathogen, the total copper content of *C. neoformans*, its copper quota, is not known. It is also not clear how the pathogen responds to variable copper conditions either *in vitro* or under conditions encountered during host infection. To address these roles of copper acquisition and complement the genetic studies of the fungus, we analyzed the intracellular copper content of *C. neoformans* by inductively coupled plasma mass spectrometry (ICP- MS) under a broad range of copper conditions *in vitro*, as well as during infection *in vivo*. We find that under typical culture growth conditions *in vitro*, *C. neoformans* sequesters as much as an order of magnitude more copper than other fungi such as *Saccharomyces cerevisiae*. In addition, *C. neoformans* is able to grow and maintain a minimal copper quota under conditions of severe copper depletion, and rapidly accrues the metal to significantly higher levels when grown in copper-supplemented media. Strikingly, this facile expansion of the cellular copper quota is a phenotype also observed *in vivo* in the mammalian host: wild-type *C. neoformans* isolated from infected mouse brain accumulates copper to levels over 50 times the minimal quota. Taken together, these studies indicate that extraordinary levels of copper acquisition are an important phenotype of this human pathogen.

RESULTS

***C. neoformans* maintains a fixed copper quota when grown under copper deficient and replete conditions**

Given the apparent importance of copper in *C. neoformans*, we examined its ability to acquire copper when both the total copper content of the medium and the chemical activity, or free copper levels, were severely restricted. For these experiments, the copper chelator bathocuproine disulfonic acid (BCS) was added in two different concentrations to normal YNB media (where the total copper concentration ranges from 25–33 nM) as well as media supplemented with copper to a level of 100 nM Cu. The copper content of *C. neoformans* was then analyzed by ICP-MS and compared cells grown in YNB media with no added copper. BCS chelates Cu (I) copper in a 2:1 ratio and the dissociation constant is estimated to be approximately 10^{-20} ;^{34,33} thus both the growth media containing total copper at 25–33 nM and BCS at 1 mM or 1 μ M correspond to significantly restricted growth conditions.

The resulting analysis showed that intracellular copper concentrations in *C. neoformans* were not significantly different under the two growth conditions, $4.6 \pm 1.2 \times 10^5$ atoms/cell and $3.4 \pm 1.2 \times 10^5$ atoms/cell, respectively. These numbers were similar to the number of copper atoms accrued by cells grown in media not supplemented with copper chelators, $3.2 \pm 0.6 \times 10^5$ atoms of copper per cell. One-way ANOVA with a Tukey post-hoc analysis revealed p-values > 0.05 , suggesting no significant difference between any of these values (Fig. 1). The average of these values represents the minimal copper quota for *Cryptococcus neoformans*. Given a minimum of six doubling times, we calculate that less than two percent of the original maternal copper bolus is passed on to offspring and conclude that the majority of copper in these cells has been acquired from the growth medium. The fact that there is little difference in copper content when grown under copper-replete and severe copper-depleted conditions indicates that this organism has robust copper homeostasis systems for maintaining a minimal copper quota.

***C. neoformans* can accumulate copper to concentrations greater than those in the surrounding media**

To study the effects of copper replete growth conditions, wild-type *C. neoformans* cells were grown in YNB media containing a range of copper concentrations and analyzed by ICP-MS. The YNB media ranged from 25–33 nM when no additional copper was added to 30 μ M of total copper with copper supplementation. Across this range, the *C. neoformans* sequestered additional copper and plateaus at levels that are two to three orders of magnitude times the minimal quota (Fig. 2). Thus total cellular copper levels increased with increasing extracellular copper in the media. Cell volumes did not significantly change despite changing extracellular copper concentration, resulting in an average cell volume of $1.3 \pm 0.04 \times 10^{-14}$ L (Fig. S1, Table S1). The uses of EDTA and BCS washes before analysis suggest that the total copper content measured here most likely corresponds to intracellular and not exogenous copper from residual media contamination in the analysis sample (Fig. S2). No significant trend was observed in the iron and zinc content of the cells (Fig. S3, Table S2). The copper content at the very highest levels of added copper is higher than observed in our previous study,¹⁶ as the cells in the current study had rested before being prepared for analysis, meaning they were stationary phase and therefore more hypoxic. We conclude that the absolute copper content of cells grown with excess copper in the medium may vary, presumably due to changes in culture conditions or growth phase.

Copper quota of *C. neoformans* is higher than that of *S. cerevisiae*

We next tested whether high levels of copper accumulation was a typical feature of fungal cell growth and compared the copper in *C. neoformans* and the non-pathogenic yeast *Saccharomyces cerevisiae*. To minimize variances due to different media compositions, both of the cell types were grown in YPD media, which contains ~400 nM copper as analyzed by ICP-MS (data not shown). While *S. cerevisiae* contains $1.3 \pm 0.2 \times 10^6$ atoms of copper per cell,³⁴ the *C. neoformans* cells accumulated almost three times that amount of copper per cell, i.e. $3.7 \pm 0.7 \times 10^6$ (Fig. 3). This pathogen clearly accumulates more copper than brewers yeast when grown in culture. To further elucidate whether *C. neoformans* also accumulates copper to high levels when growing with in the cell host and test the copper homeostasis pathways involved we next examined fungal isolates from mouse models.

Elevated pathogen copper concentration is dependent upon *CUF1* in vivo

We utilized ICP-MS to analyze both wild-type and *cuf1Δ* deletant cells that had been extracted from mouse brain tissue following 1 and 2 weeks of infection, respectively. The wild-type cells were found to contain $1.8 \pm 0.4 \times 10^7$ atoms/cell of copper; the cells are able to sequester almost 50 times their minimal metal quota (Fig. 4a). This value is in good agreement with the copper content reported in parallel experiments.¹⁶ As *cuf1Δ* cells contained $1.1 \pm 0.3 \times 10^7$ atoms/cell, both of the cell types contained similar numbers of copper atoms. Intriguingly, the cell sizes were drastically different, resulting in a significantly lower total copper concentration (i.e. moles of copper per unit volume) than observed for the wild-type cells (Fig. 4b). With a volume of $1.6 \pm 0.4 \times 10^{-14}$ L, the total copper concentration of wild-type corresponded to $1.9 \pm 0.7 \times 10^{-3}$ mol/L copper. This is almost 6-times the total copper concentration found for *cuf1Δ* cells, which had $3 \pm 2 \times 10^{-4}$ moles/L in $5.3 \pm 0.9 \times 10^{-14}$ L cell volume (Fig. 4c, d). A two-tailed t-test determined these concentration differences to be significant ($p = 0.024$). To test whether the differences in fungal copper concentrations were due to variances in brain copper concentration, brain tissue from each set of mice was analyzed to determine copper concentration. ICP-MS analysis of brain tissue from wild-type and *cuf1Δ* infected mice revealed that both tissues contained equivalent concentrations of copper, $3.1 \pm 0.9 \times 10^{-3}$ and $3.1 \pm 0.2 \times 10^{-3}$ mg Cu per gram tissue wet weight (Fig. 4d). Thus, we ascribe the difference in copper content to differences between WT and mutant physiology within the host.

To further investigate what factors might trigger this change in size in *C. neoformans*, the volume was measured on wild-type cells grown in a variety of copper concentrations. While increasing copper in the media did not result in any significant change in the cell size, addition of the copper chelator BCS resulted in a dramatic increase in the cell volume (Fig. S1). BCS was added in both 1 μ M and 1 mM concentrations to media containing approximately 70 nM Cu, resulting in a severely copper limited environment for cell growth. When grown across a range of copper concentrations, the wild-type had an average cell volume of $1.4 \pm 0.04 \times 10^{-14}$ L, almost 10-fold smaller than the $1.4 \pm 0.1 \times 10^{-13}$ L and $1.2 \pm 0.1 \times 10^{-13}$ L cells from media with 1 μ M and 1 mM BCS, respectively (Fig. 5). These data suggest that the volume expansion of the cells can be induced under copper starvation. Given the abundance of copper enzymes such as cytochrome c oxidase in mitochondria, we next looked for differences between the WT and mutant in this organelle.

CUF1-deleted cells harbor more mitochondria than wild-type

The mitochondria-specific probe MitoTracker Green was used to visualize mitochondria in both wild-type and *cuf1Δ* cells grown in culture. Confocal microscopy allowed counting of individual mitochondria per cell, while an average mitochondrial fluorescence from a large population of cells was determined using flow cytometry. Per cell, the wild-type was observed to contain 34 ± 2 mitochondria, while the *cuf1Δ* cells had over twice as many, 73

± 3 ($p < 0.001$, $N = 50$ Fig. 5a). Flow cytometry analysis of MitoTracker treated cells corroborated a doubling of the number of mitochondria; the average mitochondrial fluorescence of *cuf1Δ* cells was $2.9 \pm 0.2 \times 10^3$, as compared to $1.37 \pm 0.05 \times 10^3$ for the wild-type ($p = 0.0016$, Fig. 6c). To test whether the mitochondria in the copper starved mutant strain have any alteration in mitochondrial size or morphology, we examined these strains at the ultrastructural level by electron microscopy. Scanning transmission electron microscopy (STEM) studies revealed that even though the mutant strain has twice the mitochondria as wild-type, there are no noticeable morphological differences (Fig. 6d).

DISCUSSION

The metal quota for an organism is, by definition, the total metal content of that cell, regardless of subcellular distribution or chemical speciation. As cellular requirements for a metal change, perhaps as a function of differentiation or environmental stressors, the organism can adjust its quota to suit those requirements. The minimal quota for survival is established by analyzing total intracellular metal content after growth in a metal-deplete medium. *E. coli* grown in a metal replete media accumulate twice as much zinc as those in grown in zinc-depleted minimal media, but this change in zinc is also accompanied by a proportional increase in volume.²⁰ Organisms that have robust metal homeostasis systems frequently maintain a fixed number of moles of metal per unit volume, in spite of moderate changes in extracellular metal concentration; this same phenomenon has been observed in other cell types, including *S. cerevisiae*.³⁵ While increased metal availability beyond a threshold value may result in increasing metal content, the minimal metal quotas, i.e. determined under metal limitation, are typically maintained a very narrow range.

Our results show that *C. neoformans* avidly sequesters copper from its environment and supplements the minimal intracellular copper quota as the available concentration of copper is increased in the growth medium (Fig. 2), a phenomenon that has been encountered in some but not all prokaryotic systems.^{18,36} Interestingly, as the number of Cu atoms increases in a wild type *C. neoformans* cell we find there is no change in cell volume, and thus there is an increase in total intracellular copper concentration. This increase in concentration stands in contrast to what is observed in *E. coli*³⁷ or *S. cerevisiae*.³⁵ In light of the tightly regulated concentrations in other organisms, the ability of *C. neoformans* to accrue such large numbers of metal atoms beyond the minimal quota without a change in volume is remarkable. Recent genetic studies shed light on the physiological mechanisms at play. Theile and co-workers report that the *CUF1* locus in *C. neoformans* also regulates metallothionein-like genes which in several fungal species are associated with copper sequestration.³¹ Additionally, a recent study found increased expression of the *CMT1* and *CMT2* metallothioneins of a *ctr4Δ* mutant that was incubated in high copper media, as compared to the wild-type that was similarly exposed, further corroborating that CTR4, CUF1, and metallothioneins may be acting together to regulate copper toxicity in *C. neoformans*.¹⁶

When compared to the non-pathogenic yeast *S. cerevisiae* grown in the nutrient-rich media YPD, we observed that *C. neoformans* had already started to accrue copper from the media above the concentrations found in copper replete media (Fig. 3). *C. neoformans* has a minimal copper quota of $3.7 \pm 0.4 \times 10^5$ atoms/cell. This value is similar to but lower than that of *S. cerevisiae*, which is reported as 8.3×10^5 atoms/cell;³⁴ however, *C. neoformans* is able to significantly surpass this copper level as copper availability in the media increases, while *S. cerevisiae* does not undergo as great of an increase in intracellular concentration despite the availability of additional copper in YPD media. These elevated copper levels may be partly due to the obligate aerobe nature of *C. neoformans*; while energetically unfavorable in comparison, the facultative anaerobe *S. cerevisiae* is less reliant on

mitochondrial processes because it can also ferment without oxygen. Indeed, the hypoxic-sensing transcription factor, Sre1, has recently been shown to be required for virulence of *C. neoformans*³⁸ and, interestingly, is involved in the hypoxic-dependent induction of the copper transporter, Ctr4.⁴¹ With an increased dependence on mitochondrial function, efficient metal accumulation would appear to be essential for the fitness of *C. neoformans*, especially in oxygen-restricted environments such as the brain.

How well a pathogen can adapt to external stress is likely marked by its ability to maintain homeostasis over a number of nutrients and cofactors that are important components of the stress response. Copper availability is thought to be limited by the host at some point during infection and this would lead to a copper limitation signal in pathogens such as *C. neoformans*.¹⁵ To overcome this type of stress, the fungus must preserve basal levels of copper to enable essential physiological processes and more avidly acquire copper from the host in order to divide. Even under growth conditions where copper is severely limited through a combination of low total copper concentrations and the addition of a high affinity copper chelating agent, *C. neoformans* was capable of growth and cell division in a manner that sustains its minimal metal quota (Fig. 1). By efficiently maintaining copper homeostasis, the *C. neoformans* likely gains an advantage over its host during competition for this essential metal.

The story of copper in this pathogen becomes even more interesting in cells isolated from the brain tissue of infected mice. The *C. neoformans* cells isolated from brain accumulate 50-fold copper over the minimal quota defined by growth in cell culture conditions of limited copper availability (Fig. 4). To study this in greater detail, mice were also infected with a *cuf1Δ* strain of *C. neoformans*. Cuf1 is a metalloregulatory protein that induces expression of copper trafficking genes when cells are grown in copper deficient conditions³⁹ and apparently induces expression of MT-like genes when copper is in excess.³¹ These *cuf1Δ* mutant cells exhibit decreased virulence in mice and decreased dissemination to the CNS.¹⁵ Despite lacking this copper regulator, the *cuf1Δ* cells contained the same number of copper atoms as the wild-type cell (Fig. 4) in a much larger cell volume. Growth of *cuf1Δ* cells *in vivo* leads to nearly a 3-fold larger volume than those grown in culture. The difference in cell size between the wild-type and mutant cells isolated from mice is also significant, resulting, on average, in a 6-fold lower total copper concentration in the *CUF1*-deleted cells. This inflation in size can be replicated in cells grown in culture; under copper starvation growth conditions, wild-type *C. neoformans* were ten times the size of those grown in media without the copper chelator (Fig. 5), despite being able to maintain a minimal quota. The reproducible change in size suggests a copper-dependent phenotype, which may be induced in the Cuf1 deleted cells during infection. Thus, the difference in both size and copper content in the *cuf1Δ* cells originates in a genotypic microbial response to a common environment.

Consistent with this larger cell size, the *cuf1Δ* strain of *C. neoformans* also has an increased number of mitochondria, as detected by fluorescent mitochondrial markers. On average, the mutant strain has twice the mitochondria as the wild-type without any noticeable morphological changes as visualized with scanning transmission electron microscopy (STEM) (Fig. 6). The greater numbers of mitochondria may represent a compensatory attempt to survive under restricted copper concentrations, suggesting an advantage for this strategy as the pathogen adapt to energy demands and oxidative stress encountered within the host: both of these stresses can induce mitochondrial biogenesis).⁴⁰ However, with a lower copper concentration to draw from, this increase in mitochondria may then result in a lower mitochondrial copper concentration as compared to the wild-type. Thus, the decreased virulence of the *cuf1Δ* strain reported previously may be due to an incomplete ability to

acquire the minimal copper concentration required to support mitochondrial function under the hostile environment of the host.

EXPERIMENTAL

Preparation of chelexed and reconstituted YNB media

YNB culture media was prepared by combining 6.7 g/L Yeast Nitrogen Base without amino acids (BD, Franklin Lakes, NJ) and 20g/L Dextrose (Fisher Scientific, Pittsburgh, PA) in ultra-pure laboratory grade water (Millipore, Billerica, MA). 5 g/L of regenerated Chelex-100 Resin (Bio-Rad Laboratories, Hercules, CA) was added and the solution was stirred overnight, at which point the resin was removed through a sterile filter.

Inductively coupled plasma mass spectrometry (ICP-MS) was used to measure Mg, Ca, Fe, Ni, Cu, and Zn content in the chelexed and unchelexed YNB media. The media was diluted with ultra-pure laboratory grade water (Millipore, Billerica, MA), using internal standards as described below. Trace metal grade nitric acid (Fisher Scientific, Pittsburgh, PA) was added to the standards and samples to a final concentration of 2% w/v.

Concentrated stock solutions of MgSO_4 , CaCl_2 , FeCl_3 , NiSO_4 , ZnSO_4 , and CuSO_4 were measured by weight into ultra-pure laboratory grade water (Millipore, Billerica, MA) and used to reconstitute chelexed YNB to metal levels found in prechelexed media. Desired copper concentrations were similarly achieved by addition of aliquots of concentrated CuSO_4 dissolved in ultra-pure water.

Fungal strains and growth media

The *C. neoformans* ATCC 208821 (H99) strain was a generous gift of J. Perfect, Duke University, Durham, North Carolina, USA. The strain has been maintained as a frozen stock since acquisition in 1998 in both the author's laboratory as well as at ATCC. Strains were grown in YPD broth (2% glucose, 1% yeast extract, 2 % Bactopeptone) or on YPD agar. The *cuf1Δ* mutant of a *C. neoformans* as well as growth conditions were previously described.¹⁵

Culturing conditions of *C. neoformans* for ICP-MS analysis

In preparation for ICP-MS analysis, *C. neoformans* strains were grown for 24 hours on YPD agar and were inoculated into YPD broth and incubated for 37°C for 16 h. The OD_{600} was determined by spectrophotometry and each culture was washed in ASN media and resuspended to an OD_{600} of 0.1 in YNB media containing predetermined concentrations of copper, prepared as described previously. 25 mL cultures were grown in designated YNB media in Erlenmeyer flasks and incubated at 37°C with shaking at 250 rpm for 48–72 hours.

Isolation of *C. neoformans* from brain tissue

Sets of 10 Swiss albino mice (Harlan) were inoculated i.v. with 10^6 cfu of *C. neoformans* and the brain tissue was extracted six days post-infection (H99) or 14 days post-infection (*Cuf1Δ*). All mice experiments were done at the University of Illinois in compliance with the guidelines of the UIC institutional Animal Care and Use Committee. Brain tissue was homogenized in sterile distilled water and incubated with Proteinase K (60 mg/ml) and SDS (0.1%) at 37°C for 30 min. Following incubation the homogenate was dispensed onto a sucrose solution (70% w/v) and centrifuged for 25 min at 3000 rpm. The *C. neoformans* cell pellets were harvested, washed twice, resuspended in sterile distilled water, and analyzed by ICP-MS.

ICP-MS analysis of *C. neoformans*

ICP-MS was used to measure the copper content of *C. neoformans*. After growth in YNB media supplemented with the appropriate copper concentration or in YPD, the cells were transferred to acid-washed Teflon tubes. The cells were washed twice in YNB supplemented with 1mM EDTA and once in ultra-pure laboratory grade water (Millipore, Billerica, MA) with 1mM EDTA. After the washes, the tubes were uncapped and dried at 70°C overnight. The cells were then digested in neat trace metal grade nitric acid (Fisher Scientific, Pittsburgh, PA) for 30 minutes at 85°C. The acid solution was diluted with ultra-pure laboratory grade water (Millipore, Billerica, MA). An internal standard mixture of Sc, Tb, Y, In, and Bi (CPI International, Santa Rosa, CA) was added to this solution. Standards between 0 and 90ppb were made using a mixed element solution (CPI International). The standards and samples all contained a final concentration of 2% w/V trace metal grade nitric acid (Fisher Scientific, Pittsburgh, PA). All measurements were performed on an XSeries II ICP-MS instrument (Thermo Scientific, Waltham, MA).

Isolates of *C. neoformans* extracted from brain tissue were analyzed by ICP-MS to establish metal content. The extracted cell suspension was transferred into acid-washed Teflon tubes, and the tubes uncapped and dried at 70°C overnight. The cells were digested in neat trace metal grade nitric acid (Fisher Scientific, Pittsburgh, PA) for 30 minutes at 85°C. The acid solution was diluted with ultra-pure laboratory grade water (Millipore, Billerica, MA) and analyzed using internal standards as described above.

The copper content of mouse brain tissue was determined by ICP-MS as described above with the following modifications. Samples were prepared by digesting brain tissue in 500 μ L of concentrated trace metal free grade nitric acid (Fisher Scientific, Pittsburgh, PA) in capped, metal-free falcon tubes for two hours at 60°C. At 20 minute intervals during the digestion, the sample tubes were vortexed and vented in a fume hood. After two hours, the digests were filtered through a 0.45 μ m polytetrafluoroethylene (PTFE) filter into a fresh metal-free falcon tube. For ICP-MS analysis, a portion of the filtered digest was diluted with ultra-pure laboratory grade water ultra-pure laboratory grade water (Millipore, Billerica, MA) and analyzed as described above.

Measurement of cell volume

Cell volumes were determined by digitally measuring the diameters of ten isolated cells per sample and converting each to a volume, assuming approximately spherical shape. The diameter measurements did not include the cell capsule. Images of the cells were taken on a Nikon e600 epifluorescent microscope (Nikon Instruments Inc., Melville, NY) with a 100x objective using a CoolSNAPHQ cooled CCD camera (Photometrics, Surrey, BC). Diameter was obtained from the images with Metamorph software (Universal Imaging Corp., Downingtown, PA). Average volume for each sample was determined by averaging the volumes of all ten cells. Average volume for each cell type was determined by taking the average of all of the sample average volumes for that cell type.

Mitochondrial measurements

Mitotracker Green FM (Invitrogen, Carlsbad, CA) was used to label the mitochondria in *C. neoformans*. Cells were grown in YPD broth at 30°C for 16 h, harvested, and resuspended in 10mM HEPES buffer (pH 7.4) containing 5% glucose to a final concentration of 1×10^6 cells/mL. Mitotracker Green FM was added to the suspension at a final concentration of 100nM. The cells were incubated at room temperature for 30 minutes, washed once with water, and resuspended in water to a concentration of 1×10^6 cells/mL. Mitochondria were directly examined for fluorescence by confocal microscopy (Leica DMI 6000B). Representative mitochondrial numbers per cell for each strain were determined by direct

counting of the mitochondria contained in 50 randomly selected cells, and an average mitochondrial number was calculated. Mean mitochondrial fluorescence was compared using flow cytometry, performed with an LSR Fortessa flow cytometer (BD Biosciences, Franklin Lakes, NH). Fluorescent signal was collected using the 530/30 nm BandPass Filter and 505 LP LongPass Mirror. FITC Mean fluorescence was determined by the FACS Diva Software (BD BioSciences) from 1×10^4 cells per sample.

Mitochondrial Imaging by STEM

C. neoformans, wild-type and *cuf1Δ*, were grown in YPD broth for 24 hours. Cells in media were transferred into 15 mL centrifugation tubes and kept at room temperature until they formed a pellet. The supernatant was aspirated and the cell pellets were resuspended in a small volume of medium and mixed with 2% low melting point agarose (Fisher Scientific, Pittsburgh, PA) at a ratio of 1:1. After the agarose solidified, small sections were dissected and transferred into specimen carriers for the high-pressure freezer (EM PACT2, Leica), mounted, and high-pressure frozen. Freeze substitution was carried out in a programmable freeze substitution device (EM AFS2, Leica): the frozen carriers with the samples were collected in liquid nitrogen, then transferred into a solution of 0.2 % OsO₄ and 0.1 % UA and 5 % water in acetone at -85°C ,⁴¹ and kept for 72 hours. Subsequently, the temperature was raised continuously to 0°C in 16 hours. The samples were kept at 0°C for 3 hours, rinsed in acetone for 30 minutes, and separated from the carriers in fresh acetone. The samples were infiltrated with Spurr resin diluted with acetone 1:1 overnight at 4°C . The next day, samples were transferred into Spurr resin and infiltrated for 8 hours at 4°C . Finally the samples were brought to room temperature, transferred into fresh resin in embedding molds and cured for 72 hours at 65°C . Ultrathin sections with golden interference color were mounted on 200 mesh copper grids, and observed in a Hitachi HD2300 STEM at an acceleration voltage of 80 keV.

Statistical Analysis

Results are reported as means $\pm$ SEM and n represents the number of independent experiments performed. Statistical analysis was performed with GraphPad Prism. One-way ANOVA, followed by Tukey's post-hoc analysis, and Student t-test were employed to test for significance between values. *P*-values ≤ 0.05 and 0.005 were considered statistically significant and highly significant, respectively.

CONCLUSION

The close association of copper quota with virulence factors of *C. neoformans* highlights the importance of copper acquisition as a key to the survival and spread of this pathogen. These results provide further insight into emerging copper homeostatic pathways in *C. neoformans* and suggest that efficient high affinity uptake and metalloregulatory systems provide an advantage against host defenses. While the full role of the higher copper quota in the pathogenicity of *C. neoformans* remains to be established, the current findings open the door to new types of pharmacological intervention. If pathogen copper acquisition is truly challenged inside the host, then this fact can be exploited in the development of new therapeutics to treat and control the spread of this deadly pathogen.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

This research was funded, in part, by the following grants: GM038784, GM038784-22S1, and GM54111. This study was also partly supported by United States Public Health Service Grant NIH-AI45995, AI49371 and by the Intramural Research Program of the NIH, NIAID. ICP-MS metal analysis was performed at the Northwestern University Quantitative Bioelemental Imaging Center generously supported by NASA Ames Research Center NNA04CC36G. Electron microscopy work was performed in the Electron Probe Instrumentation Center (EPIC) facility of NUANCE Center (supported by NSF-NSEC, NSF-MRSEC, Keck Foundation, and the State of Illinois) at Northwestern University. Also, we gratefully acknowledge support by the Northwestern University Quantitative Bioelemental Imaging Center, generously funded by the Keck Foundation and the Chicago Biomedical Consortium sponsor #C2006-00997, NU #CNVR48279.

References

1. Park BJ, Wannemuehler KA, Marston BJ, Govender N, Pappas PG, Chiller TM. Estimation of the current global burden of cryptococcal meningitis among persons living with HIV/AIDS. *AIDS*. 2009; 23:525–30.10.1097/QAD.0b013e328322ffac [PubMed: 19182676]
2. Husain S, Wagener MM, Singh N. Cryptococcus neoformans infection in organ transplant recipients: variables influencing clinical characteristics and outcome. *Emerg Infect Dis*. 2001; 7:375–81. [PubMed: 11384512]
3. Yao Z, Liao W, Chen R. Management of cryptococcosis in non-HIV-related patients. *Med Mycol*. 2005; 43:245–51. [PubMed: 16010851]
4. Pyrgos VSAE, Steiner CA, Prevots DR, Williamson PR. Epidemiology of Cryptococcal meningitis in the U.S.: 1997–2009. *PlosOne*. 2013 (in press).
5. Pathogenic yeasts. Springer; New York: 2009.
6. Buchanan KL, Murphy JW. What makes Cryptococcus neoformans a pathogen? *Emerg Infect Dis*. 1998; 4:71–83. [PubMed: 9452400]
7. Prentice AM, Ghattas H, Cox SE. Host-pathogen interactions: can micronutrients tip the balance? *J Nutr*. 2007; 137:1334–7. 137/5/1334 [pii]. [PubMed: 17449601]
8. Schaible UE, Kaufmann SH. Iron and microbial infection. *Nat Rev Microbiol*. 2004; 2:946–53. nrmicro1046 [pii]. 10.1038/nrmicro1046 [PubMed: 15550940]
9. Sutak R, Lesuisse E, Tachezy J, Richardson DR. Crusade for iron: iron uptake in unicellular eukaryotes and its significance for virulence. *Trends Microbiol*. 2008; 16:261–8. S0966-842X(08)00089-9 [pii]. 10.1016/j.tim.2008.03.005 [PubMed: 18467097]
10. Huynh C, Andrews NW. Iron acquisition within host cells and the pathogenicity of Leishmania. *Cell Microbiol*. 2008; 10:293–300. CMI1095 [pii]. 10.1111/j.1462-5822.2007.01095.x [PubMed: 18070118]
11. Ratledge C, Dover LG. Iron metabolism in pathogenic bacteria. *Annu Rev Microbiol*. 2000; 54:881–941. [pii]. 10.1146/annurev.micro.54.1.88154/1/881 [PubMed: 11018148]
12. Doherty CP. Host-pathogen interactions: the role of iron. *J Nutr*. 2007; 137:1341–4. 137/5/1341 [pii]. [PubMed: 17449603]
13. Chun CD, Madhani HD. Ctr2 links copper homeostasis to polysaccharide capsule formation and phagocytosis inhibition in the human fungal pathogen Cryptococcus neoformans. *PLoS One*. 2010; 510.1371/journal.pone.0012503
14. Jiang N, Liu X, Yang J, Li Z, Pan J, Zhu X. Regulation of copper homeostasis by Cuf1 associates with its subcellular localization in the pathogenic yeast Cryptococcus neoformans H99. *FEMS Yeast Res*. 2011; 11:440–8.10.1111/j.1567-1364.2011.00733.x [PubMed: 21489137]
15. Waterman SR, Hacham M, Hu G, Zhu X, Park YD, Shin S, Panepinto J, Valyi-Nagy T, Beam C, Husain S, Singh N, Williamson PR. Role of a CUF1/CTR4 copper regulatory axis in the virulence of Cryptococcus neoformans. *J Clin Invest*. 2007; 117:794–802.10.1172/JCI30006 [PubMed: 17290306]
16. Waterman SR, Park YD, Raja M, Qiu J, Hammoud DA, O'Halloran TV, Williamson PR. Role of CTR4 in the Virulence of Cryptococcus neoformans. *MBio*. 2012; 3 mBio.00285–12 [pii]. 10.1128/mBio.00285-12

17. Linder MC, Hazegh-Azam M. Copper biochemistry and molecular biology. *Am J Clin Nutr.* 1996; 63:797S–811S. [PubMed: 8615367]
18. Pufahl RA, Singer CP, Peariso KL, Lin SJ, Schmidt PJ, Fahrni CJ, Culotta VC, Penner-Hahn JE, O'Halloran TV. Metal ion chaperone function of the soluble Cu(I) receptor Atx1. *Science.* 1997; 278:853–6. [PubMed: 9346482]
19. Labbe S, Thiele DJ. Pipes and wiring: the regulation of copper uptake and distribution in yeast. *Trends Microbiol.* 1999; 7:500–5. S0966-842X(99)01638-8 [pii]. [PubMed: 10603486]
20. Outten CE, O'Halloran TV. Femtomolar sensitivity of metalloregulatory proteins controlling zinc homeostasis. *Science.* 2001; 292:2488–92. [pii]. 10.1126/science.1060331060331 [PubMed: 11397910]
21. Brand LE. Minimum Iron Requirements of Marine Phytoplankton and the Implications for the Biogeochemical Control of New Production. *Limnology and Oceanography.* 1991; 36:1756–1771.
22. Andreini C, Banci L, Bertini I, Rosato A. Occurrence of copper proteins through the three domains of life: a bioinformatic approach. *J Proteome Res.* 2008; 7:209–16.10.1021/pr070480u [PubMed: 17988086]
23. Liu XD, Liu PC, Santoro N, Thiele DJ. Conservation of a stress response: human heat shock transcription factors functionally substitute for yeast HSF. *EMBO J.* 1997; 16:6466–77.10.1093/emboj/16.21.6466 [PubMed: 9351828]
24. Finney LA, O'Halloran TV. Transition metal speciation in the cell: insights from the chemistry of metal ion receptors. *Science.* 2003; 300:931–6. [pii]. 10.1126/science.1085049300/5621/931 [PubMed: 12738850]
25. Pena MM, Lee J, Thiele DJ. A delicate balance: homeostatic control of copper uptake and distribution. *J Nutr.* 1999; 129:1251–60. [PubMed: 10395584]
26. La Fontaine S, Quinn JM, Nakamoto SS, Page MD, Gohre V, Moseley JL, Kropat J, Merchant S. Copper-dependent iron assimilation pathway in the model photosynthetic eukaryote *Chlamydomonas reinhardtii*. *Eukaryot Cell.* 2002; 1:736–57. [PubMed: 12455693]
27. Salas SD, Bennett JE, Kwon-Chung KJ, Perfect JR, Williamson PR. Effect of the laccase gene CNLAC1, on virulence of *Cryptococcus neoformans*. *J Exp Med.* 1996; 184:377–86. [PubMed: 8760791]
28. Cox GM, Harrison TS, McDade HC, Taborda CP, Heinrich G, Casadevall A, Perfect JR. Superoxide dismutase influences the virulence of *Cryptococcus neoformans* by affecting growth within macrophages. *Infect Immun.* 2003; 71:173–80. [PubMed: 12496163]
29. Zhu X, Gibbons J, Zhang S, Williamson PR. Copper-mediated reversal of defective laccase in a *Deltavph1* avirulent mutant of *Cryptococcus neoformans*. *Mol Microbiol.* 2003; 47:1007–14. 3340 [pii]. [PubMed: 12581355]
30. Kim BE, Nevitt T, Thiele DJ. Mechanisms for copper acquisition, distribution and regulation. *Nat Chem Biol.* 2008; 4:176–85. nchembio.72 [pii]. 10.1038/nchembio.72 [PubMed: 18277979]
31. Ding C, Yin J, Tovar EM, Fitzpatrick DA, Higgins DG, Thiele DJ. The copper regulon of the human fungal pathogen *Cryptococcus neoformans* H99. *Mol Microbiol.* 2011; 81:1560–76.10.1111/j.1365-2958.2011.07794.x [PubMed: 21819456]
32. Nyhus K, Jacobson ES. Oxy2 as a transcriptional activator gene for copper uptake in *Cryptococcus neoformans*. *Med Mycol.* 2004; 42:325–31. [PubMed: 15473357]
33. Rae TD, Schmidt PJ, Pufahl RA, Culotta VC, O'Halloran TV. Undetectable intracellular free copper: the requirement of a copper chaperone for superoxide dismutase. *Science.* 1999; 284:805–8. [PubMed: 10221913]
34. Wolford, JL. Zinc Localization and Quantitation in Specialized Cells and Tissues. Northwestern University; Evanston, IL: 2006.
35. Brown, NM.; Wolford, JL.; O'Halloran, VT. Northwestern University; 2006.
36. McGann P, Hang J, Clifford RJ, Yang Y, Kwak YI, Kuschner RA, Lesho EP, Waterman PE. Complete sequence of a novel 178-kilobase plasmid carrying bla(NDM-1) in a *Providencia stuartii* strain isolated in Afghanistan. *Antimicrob Agents Chemother.* 2012; 56:1673–9. AAC.05604-11 [pii]. 10.1128/AAC.05604-11 [PubMed: 22290972]
37. Kubitschek HE. Cell volume increase in *Escherichia coli* after shifts to richer media. *J Bacteriol.* 1990; 172:94–101. [PubMed: 2403552]

38. Chun CD, Liu OW, Madhani HD. A link between virulence and homeostatic responses to hypoxia during infection by the human fungal pathogen *Cryptococcus neoformans*. *PLoS Pathog.* 2007; 3:e22. 06-PLPA-RA-0472R2 [pii]. 10.1371/journal.ppat.0030022 [PubMed: 17319742]
39. Beaudoin J, Labbe S. Copper induces cytoplasmic retention of fission yeast transcription factor *cuf1*. *Eukaryot Cell.* 2006; 5:277–92. 5/2/277 [pii]. 10.1128/EC.5.2.277-292.2006 [PubMed: 16467469]
40. Yoboue ED, Devin A. Reactive oxygen species-mediated control of mitochondrial biogenesis. *Int J Cell Biol.* 2012; 2012:403870.10.1155/2012/403870 [PubMed: 22693510]
41. Buser C, Walther P. Freeze-substitution: the addition of water to polar solvents enhances the retention of structure and acts at temperatures around –60 degrees C. *J Microsc.* 2008; 230:268–77. JMI1984 [pii]. 10.1111/j.1365-2818.2008.01984.x [PubMed: 18445157]

Figure 1.

In copper deficient and deplete conditions, *Cryptococcus neoformans* is able to maintain a minimal copper quota. Wild-type *C. neoformans* was grown in YNB media with specific copper concentrations, as indicated. For the BCS-treated controls, the indicated concentration of BCS was added to the solution of YNB growth media containing 100nM Cu. “No added copper” represents media in which copper was removed using Chelex-100 resin and no additional copper was reintroduced (n=1 for 25 nM and n=2 for 33 nM total media copper). The copper concentrations in the cells were analyzed by ICP-MS and values are reported as Mean $\pm$ SEM (n=3, each in triplicate).

Figure 2.

Cryptococcus neoformans can concentrate available copper as a function of increasing concentration in the growth medium. *C. neoformans* was grown in YNB containing a broad range of copper concentrations. Mean $\pm$ SEM is shown ($n=3$). “No added copper” represents media in which copper was removed with Chelex-100 resin ($n=2$ for 25 nM and $n=1$ for 33 nM total media copper). The minimal copper quota of the fungus, as calculated by taking the average atoms of copper from growths in 1mM and 1μM concentrations of BCS and in media with no added copper, is represented by the dashed line.

Figure 3.

Cryptococcus neoformans has a higher copper quota than *S. cerevisiae*. Wild-type *Cryptococcus neoformans* and *Saccharomyces cerevisiae* were grown in YPD media, containing ~400nM copper, and intracellular copper was analyzed with ICP-MS, as described in methods. Mean $\pm$ SEM shown (n=3 for *C. neoformans* n=6 for *S. cerevisiae*, each in triplicate).

Figure 4.

Wild-type cells can sequester large amounts of copper *in vivo*. (A) Wild-type cells isolated from infected mouse brains compared against *in vitro* assays demonstrate a high number of copper atoms per cell, as compared to those grown in culture. (B) The *cuf1Δ* cells isolated from mice have a larger average volume, as compared to the wild-type cells. Representative image shows this size difference. Scale bar of 5 μm is shown in blue. (C) Comparison of cells extracted from infected mouse brain demonstrates that both the wild-type and *cuf1Δ* mutant cells accumulate the same number of atoms of copper per cell. For wild-type and *cuf1* deleted cells, infection times in the mouse were one and two weeks, respectively. The cells were extracted and copper levels in the cells were analyzed by ICP-MS, as described in Methods and values are reported as Mean ± SEM (n=6 for WT and n=5 for *cuf1Δ*). (D) The differing cell size results in a significant 6-fold difference in copper concentration between the wild-type and *cuf1Δ* cells. **, p < 0.005 as compared to the wild-type (E) The difference in cell volume, and thereby copper concentration, is not a consequence of brain copper variances, as the brain tissue that hosted both cells contains the same copper concentration. Tissue was digested and analyzed by ICP-MS and values are reported as Mean ± SEM (n=3 and n=4 for tissue from wild-type and *cuf1Δ* infected mice, respectively)

Figure 5. Under copper deficient growth conditions, *C. neoformans* increase in size. (A) Wild-type *C. neoformans* was grown in YNB with the indicated BCS concentrations. The YNB media was analyzed to show a copper content of ~ 70 nM, assuring that the added BCS would sufficiently chelate the present copper. Cells grown with BCS show a 10-fold increase in volume as compared to cells grown in YNB media with varying copper concentrations. The cell sizes were measured as described in Methods and values are reported as Mean $\pm$ SEM (n=20 for cells grown in BCS and n=100 for cells grown in varying copper concentrations). (B) Representative image shows this size difference. Scale bar of 5μ m is shown in blue.

Figure 6.

CUF-1 deleted *C. neoformans* contains twice the mitochondria of wild-type. (A) Mitochondria in wild-type and *cuf1Δ* cells were identified and counted based on staining with the mitochondria-specific probe MitoTracker Green. Values represent the average mitochondria per cell (n=50). **, p < 0.005 as compared to the wild-type. (B) Representative image of cells stained with MitoTracker Green shows a higher presence of mitochondria in the mutant cell, as compared to wild-type. (C) Mitochondrial fluorescence from MitoTracker Green was also analyzed using flow-cytometry. Average fluorescence is reported from 10,000 events (n=3). **, p < 0.005, as compared to the wild-type. (D) Despite increased mitochondrial number, no morphological changes were detected between the

cuf1Δ and wild-type cells using an HD2300 STEM. One mitochondrion from each image is labeled with an m, and the nucleus is represented by the word nucleus.