Role of *CTR4* in the Virulence of *Cryptococcus neoformans*

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ABSTRACT **While research has identified an important contribution for metals, such as iron, in microbial pathogenesis, the roles of other transition metals, such as copper, remain mostly unknown. Recent evidence points to a requirement for copper homeostasis in the virulence of *Cryptococcus neoformans* based on a role for a *CUF1* copper regulatory factor in mouse models and in a human patient cohort. *C. neoformans* is an important fungal pathogen that results in an estimated 600,000 AIDS-related deaths yearly. In the present studies, we found that a *C. neoformans* mutant lacking the *CUF1*-dependent copper transporter, *CTR4*, grows normally in rich medium at 37°C but has reduced survival in macrophages and attenuated virulence in a mouse model. This reduced survival and virulence were traced to a growth defect under nutrient-restricted conditions. Expression studies using a full-length *CTR4*-fluorescent fusion reporter construct demonstrated robust expression in macrophages, brain, and lung, the latter shown by *ex vivo* fluorescent imaging. Inductively coupled mass spectroscopy (ICP-MS) was used to probe the copper quota of fungal cells grown in defined medium and recovered from brain, which suggested a role for a copper-protective function of *CTR4* in combination with cell metallothioneins under copper-replete conditions. In summary, these data suggest a role for *CTR4* in copper-related homeostasis and subsequently in fungal virulence.**

IMPORTANCE ***Crytococcus neoformans* is a significant global fungal pathogen, and copper homeostasis is a relatively unexplored aspect of microbial pathogenesis that could lead to novel therapeutics. Previous studies correlated expression levels of a Ctr4 copper transporter to development of meningoencephalitis in a patient cohort of solid-organ transplants, but a direct role for Ctr4 in mammalian pathogenesis has not been demonstrated. The present studies utilize a Δ*ctr4* mutant strain which revealed an important role for *CTR4* in *C. neoformans* infections in mice and relate the gene product to homeostatic control of copper and growth under nutrient-restricted conditions. Robust expression levels of *CTR4* during fungal infection were exploited to demonstrate expression and lung cryptococcal disease using *ex vivo* fluorescence imaging. In summary, these studies are the first to directly demonstrate a role for a copper transporter in fungal disease and provide an *ex vivo* imaging tool for further study of cryptococcal gene expression and pathogenesis.**

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Cryptococcus neoformans is a major fungal pathogen that causes fatal meningoencephalitis predominantly in immunocompromised hosts, including AIDS patients, transplant recipients receiving conditioning regimens, and cancer patients on chemotherapy. Worldwide, *C. neoformans* accounts for a large proportion of AIDS-related deaths (1). Infection is believed to be acquired through the inhalation of yeast cells into the lungs. After inhalation, the alveolar macrophages provide an initial cellular defense, activated by a combination of innate and acquired immune responses (2). It is proposed that *Cryptococcus* uses macrophage residency as an efficient mechanism to evade further host immune attack and enable systemic bloodstream infection and dissemination (3).

Copper is a redox-active transition metal that can serve as an electron transfer intermediate for catalytic enzyme activity (4). However, this same redox property makes copper a toxic element when in excess, reacting with oxygen to produce damaging hydroxyl radicals (5) and, thus, requiring tight homeostasis within the cell (6, 7). Invading pathogens enter into this highly regulated environment of the host and must compete for limited nutrients, including transition metals, such as iron and copper, to colonize and replicate during infection (8). The importance of copper homeostasis in pathogens has been suggested in *C. neoformans* by the dependency of two virulence factors in the organism, a Cu/Zn superoxide dismutase (9) and a copper-containing laccase enzyme (10), and more recently by a role for the copper transporter/chaperone, *CCC2*, deletion of which results in attenuated virulence (11). The importance of copper to pathogenesis is also suggested by an essential role for the copper-containing cytochrome *c* oxidase (12) in this obligate aerobe as well as a role in

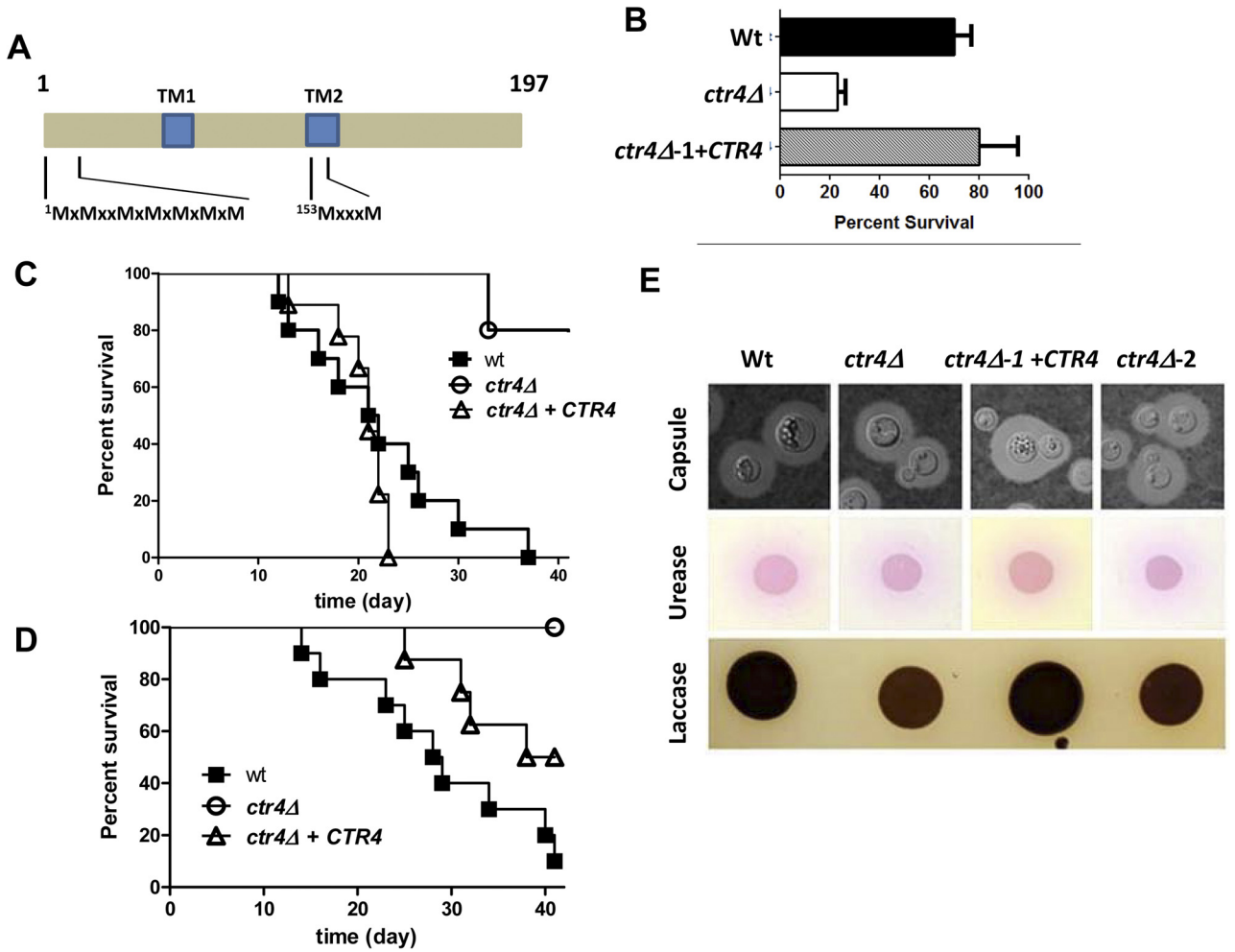

FIG 1 Reduced survival of a Δ*ctr4* mutant in macrophages and attenuated virulence in mice. (A) Schematic of the Ctr4 protein sequence, including MxM copper binding sites and TM1 and TM2 transmembrane domains. (B) RAW264.7 cells were infected with wt, Δ*ctr4* mutant, and a *CTR4* complemented strain, and percent survival was determined as the ratio of fungal CFU at 24 versus 2 h postinfection ($n = 3$ independent experiments; ±standard error of the mean [SEM]). (C) Mice were infected intravenously with 10^3 CFU of the indicated *C. neoformans* strains ($n = 10$ mice each group). (D) Mice were infected intranasally with 10^6 CFU of the indicated *C. neoformans* strains ($n = 10$ mice each group). (E) Indicated strains were incubated 2 days on agar containing a 1:10 dilution of Sabouraud medium (1:10 SAB) at 37°C and observed by India ink microscopy for capsule, incubated in Christensen's media overnight at 37°C for urease, or incubated in 0.05% glucose, asparagine salts, pH 7.4, at 37°C for laccase activity.

virulence (10), which is dependent on a vesicular chloride channel and proton pump for copper metalation and virulence (13, 14). In addition, copper homeostasis may play a more widespread role in virulence through its effects on Fe^+ acquisition (15), as copper is a substrate for the high-affinity Fe^+ transporter Fet3 (16). The importance of copper in chemotherapeutics is suggested by recent studies proposing microplusin, a copper-chelating antimicrobial peptide, as a potential therapy against *C. neoformans* (17).

A Cu-sensing transcription factor, Cuf1, is required for cryptococcal virulence in mice and fungal proliferation in the human brain (18). Cuf1 acts as a dual sensor of copper levels, responsible for expression of a set of copper-specific copper transporters, *CTR1* and *CTR4*, at low copper concentrations and two metallothioneins, *CMT1* and *CMT2*, at high copper concentrations (19). Metallothioneins protect against toxic levels of copper at high copper concentrations, the latter as evidenced by defective growth of a cryptococcal Δ*cmt1* Δ*cmt2* double mutant in the presence of 1 mM Cu (19). The copper transporter *CTR1* (CNAG_07701) was originally named *CTR2* and linked copper homeostasis to polysaccharide capsule formation and phagocytosis (20) but was renamed in a subsequent study due to its greater homology and more similar expression profile to the *Staphylococcus cerevisiae CTR1* gene (19). On the other hand, the gene currently designated *CTR2* (CNAG_01872) does not exhibit copper-dependent expression in either *S. cerevisiae* or *C. neoformans* (19). *CTR4* expression was initially described in *C. neoformans* as a copper-dependent reporter (21), and expression of this Cuf1-dependent gene was found to be increased during macrophage and brain infection. Furthermore, *CTR4* was used as a marker of Cuf1 activity, and its expression level in clinical fungal isolates was found to be associated with increased propensity to cause meningoencephalitis in a cohort of solid organ transplant patients (18). However, while both *CTR1* and *CTR4* contain consensus copper binding elements and two transmembrane domains typical of copper transporters (Fig. 1A) and both successfully complemented a copper transporter-defective *S. cerevisiae* Δ*ctr1* Δ*ctr3* strain, *CTR4*

was found to be dispensable for growth in copper-deficient, nutrient-rich medium (19).

Thus, to further characterize a possible direct association between *CTR4* and virulence in *C. neoformans*, we investigated the expression and virulence of *CTR4* using a set of Δ*ctr4* knockout cryptococcal strains and a robust *CTR4* reporter strain of *C. neoformans*. These studies revealed normal growth in rich medium as reported previously (19) but a severe growth defect of the Δ*ctr4* mutant under nutrient-limiting conditions. *CTR4* deletion also led to a reduction in survival of *C. neoformans* in macrophages and attenuated virulence in mice. These data thus support a relationship between the *CTR4* copper transporter and nutrient-restricted growth and virulence in *C. neoformans*.

RESULTS

Attenuated virulence of a Δ*ctr4* mutant of *C. neoformans*. To establish a correlation between *CTR4*-dependent copper homeostasis and the ability to cause infections, we first assayed for survival of the Δ*ctr4* mutant strain in RAW264.7 cells after activation with gamma interferon (IFN-γ) for 16 h, since macrophage survival is critical for this facultative intracellular pathogen (3). Fungal cells were opsonized with an anticapsular monoclonal antibody (MAb) GXM 18B7 and incubated at a 10:1 ratio to RAW264.7 cells for 1 h, and the survival rate was calculated as a ratio of fungal CFU from lysed RAW264.7 cells at 24 versus 2 h. As shown in Fig. 1B, the Δ*ctr4* mutant exhibited a significant reduction in survival compared to that in both the wild type (wt) and the *CTR4* complemented strain, with the mutant exhibiting only approximately 20% survival, versus approximately 80% in the wt and complemented strains ($P < 0.001$). We next assessed for virulence using an intravenous mouse model which showed attenuated survival of the Δ*ctr4* mutant, compared to that of either the wt or *CTR4* complemented strain (Δ*ctr4* mutant versus wt or complemented strain, $P < 0.001$; Fig. 1C). In addition, the Δ*ctr4* mutant was compared using an intranasal mouse model in which it also displayed significant defects in virulence (Δ*ctr4* mutant versus wt or complemented strain, $P < 0.01$; Fig. 1D). The complemented strain showed a trend toward less virulence than the wt strain in the intranasal model but was not statistically significant ($P = 0.07$; Fig. 1D). These data thus demonstrate an important role for the cryptococcal *CTR4* gene in macrophage survival as well as virulence in mice.

Further studies demonstrated a minor role for *CTR4* in expression of known virulence factors of *C. neoformans*. As shown in Fig. 1E, laccase expression was reduced somewhat in the Δ*ctr4* mutant, as exhibited by reduced pigment formation in the presence of norepinephrine, and was restored in the complemented strain, as might be expected for a copper-dependent enzyme (22). Laccase expression was previously reported to be equal to that in the wild type in a previously constructed Δ*ctr4* mutant (19), which could have been due to differing conditions, such as pH (5.5 versus 7.4 in the present studies), which affects trafficking of the enzyme (23). Capsule inductions under low-nutrient conditions (1:10 Sabouraud medium), which induces capsule (24), were similar between the strains. The urease enzyme was also not affected in the Δ*ctr4* mutant, since this activity is thought to be dependent on zinc (25). However, while complete disruption of these virulence factors is well correlated with virulence (10, 26, 27, 28), small quantitative differences, such as demonstrated for laccase expression, have not been as strongly associated with differences in virulence in mice (29).

Thus, to understand further the role of *CTR4* in virulence, we examined the growth characteristics of the wt, Δ*ctr4*, and Δ*ctr4* *CTR4* complemented mutant strains of *C. neoformans* under nutrient-replete conditions as well as under nutrient limitation. Recent studies showing induction of genes involved in autophagy in macrophages (30) as well as its requirement for virulence (31) suggest that niche environments within the host may be nutrient restricted. Interestingly, while growth of the mutant was normal in the rich medium, yeast extract-peptone-dextrose (YPD) (Fig. 2, top), as reported previously (19), the Δ*ctr4* mutant exhibited a severe defect under nutrient limitation (2% glucose, asparagine salts). Supplementation with amino acids (synthetic complete medium [SC]) resulted in partial restoration of growth that was further restored by the addition of a vitamin cocktail. Interestingly, the growth defect was similar in each medium under all copper concentrations, suggesting that copper homeostasis cannot be restored in the mutant by supplementation with exogenous copper or that Ctr4 has a shared function with other cellular pathways involved in nutrient-limited growth. Growth could also not be restored by addition of a single L amino acid, by addition of higher levels of copper up to 1 mM, or by addition of a membrane-permeant copper chelator such as pyrithione to copper salts or reducing agents such as ascorbate, the latter shown to restore laccase activity in a copper metalation *C. neoformans* Δ*vph1* mutant (data not shown) (32). These data demonstrate a role for *CTR4* in growth under nutrient-limiting conditions and suggest a possible relationship of this phenotype with the ability of *CTR4* to potentiate wild-type virulence.

***CTR4*-fluorescent fusion proteins demonstrate a plasma membrane and vacuole localization.** Previous studies using a *CTR4*-green fluorescent reporter construct demonstrated copper-dependent expression, but inclusion of only a small N-terminal portion of the protein prevented studies of cellular targeting (18). Thus, to permit more robust cellular localization and expression studies, a new set of constructs was generated using a full-length *CTR4* protein expressed from the native promoter. In addition, to reduce background autofluorescence and thus improve relative expression levels, a *C. neoformans* codon-optimized mCherry was constructed, as excitation in the red spectrum is typically associated with reduced problems of autofluorescence (33). As shown in Fig. 3A, expression of the Ctr4-green fluorescent protein (GFP) in wt *C. neoformans* localized to the periphery, consistent with localization in the plasma membrane, most likely due to the presence of two canonical transmembrane domains in the new full-length Ctr4 fusion construct (Fig. 1A). To confirm the function of Ctr4 fusion proteins, Ctr4-mCherry was expressed in a Δ*ctr4* mutant, which restored growth in minimal medium (Fig. 3B) and demonstrated increased expression in media containing the copper chelator bathocuproine disulfonate (BCS) (Fig. 3C) by flow cytometry, as previously demonstrated by Northern hybridization (18, 19). The Ctr4-mCherry protein also displayed a peripheral localization suggestive of a plasma membrane protein as well as intracellular punctae, the latter colocalizing with the yeast vacuole on differential interference contrast (DIC) microscopy (Fig. 3D). Time-dependent observation revealed increasing amounts of vacuolar fluorescent signal compared to the plasma-membrane signal over time (data not shown), which has been observed during fusion protein turnover with vacuolar retention of the more stable

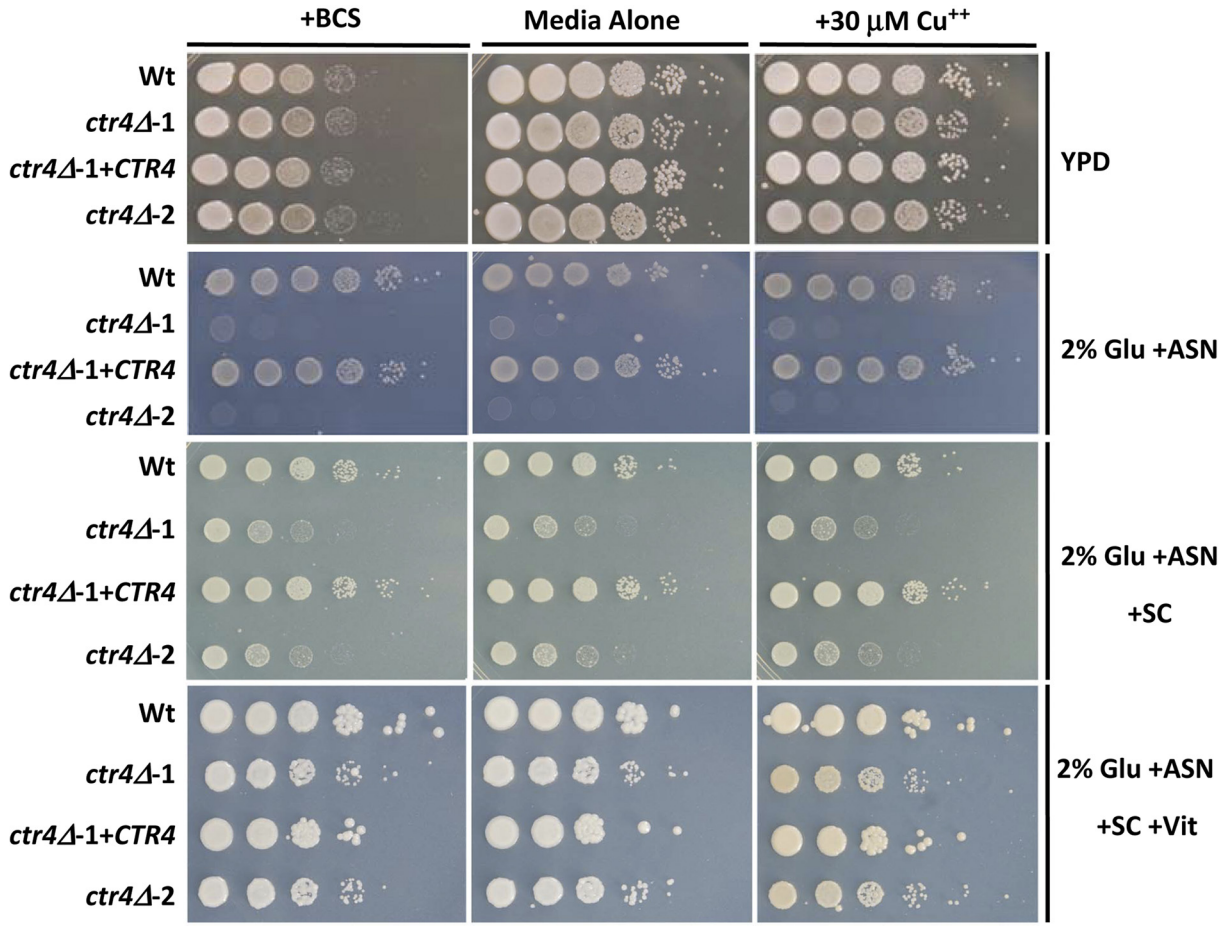

FIG 2 Reduced growth of a Δ*ctr4* mutant strain on nutrient-deficient but not nutrient-replete agar. Indicated strains were spot plated at sequential 1:5 dilutions on the indicated medium supplemented with either bovine calf serum (BCS) (100 μM) or 30 μM $CuSO_4$ and incubated at 37°C for 2 to 3 days.

fluorescent protein fragment (34). Fungi harboring the *CTR4* fusion protein were allowed to infect RAW264.7 cells and observed for 24 h, which again displayed the fusion protein in a peripheral location (Fig. 4A), suggesting that the function of *CTR4* is analogous under both *in vitro* and *in vivo* conditions. Furthermore, equivalent fungal cells recovered from lung after intravenous inoculation again displayed a peripheral location in lung (Fig. 4B), although with significant signal within yeast vacuoles, consistent with significant fusion protein turnover (and vacuolar accumulation of the stable fluorescent tag) during the longer incubation time experienced during the 9 days of infection of the animals. This construct containing the intact *CTR4* protein exhibited robust expression in the mouse with significant expression within lung tissues, which was not evident previously, using an N-terminal *CTR4* protein construct, suggesting that inclusion of the full-length protein sequence may have increased expression or stability of the fusion protein. Interestingly, the Ctr4-mCherry demonstrated exclusively a vacuolar pattern in brain (Fig. 4B) and a predominance of the vacuolar signal in lung, which could be due to greater persistence/stability of the mCherry than the GFP protein after Ctr4 expression and turnover.

Optical imaging of a cryptococcal infection in a pulmonary mouse model. Because of the robust lung expression of the new *CTR4*-mCherry construct, we sought to determine if the new strain could be used to visualize cryptococcal lung infections using optical imaging. Mice were infected with 1×10^6 CFU of the Ctr4-mCherry-expressing *C. neoformans* strain or an equivalent strain carrying an empty vector and euthanized when moribund after 9 days. The animals were then imaged simultaneously side by side using a Maestro 2 *in vivo* Imaging System (CRi, Inc., Woburn, MA). As shown in Fig. 5A, fluorescent signal could be detected in the region of the thoracic cavity of the mouse infected with the Ctr4-mCherry-expressing strain but not in the mouse infected by a congenic strain expressing empty vector (EV). To confirm that the signal was emitted from the lungs, organs were excised and reimaged. As shown in Fig. 5C, the excised lung of the *CTR4*-mCherry mouse displayed significant fluorescence signal without significant signal arising from the lungs of the control animal. The remainder of the organs demonstrated no detectable signal (data not shown). Unfortunately, the attenuation of light by the skull of the mouse prevented visualization of fluorescent signal arising from the mouse brain. These data demonstrate that optical imaging of *CTR4*-expressing *C. neoformans* is feasible and that *CTR4* is vigorously expressed during lung infection. These data also demonstrate that optical imaging can be used to noninvasively follow the progress of infection and potentially assess the response to various treatment approaches.

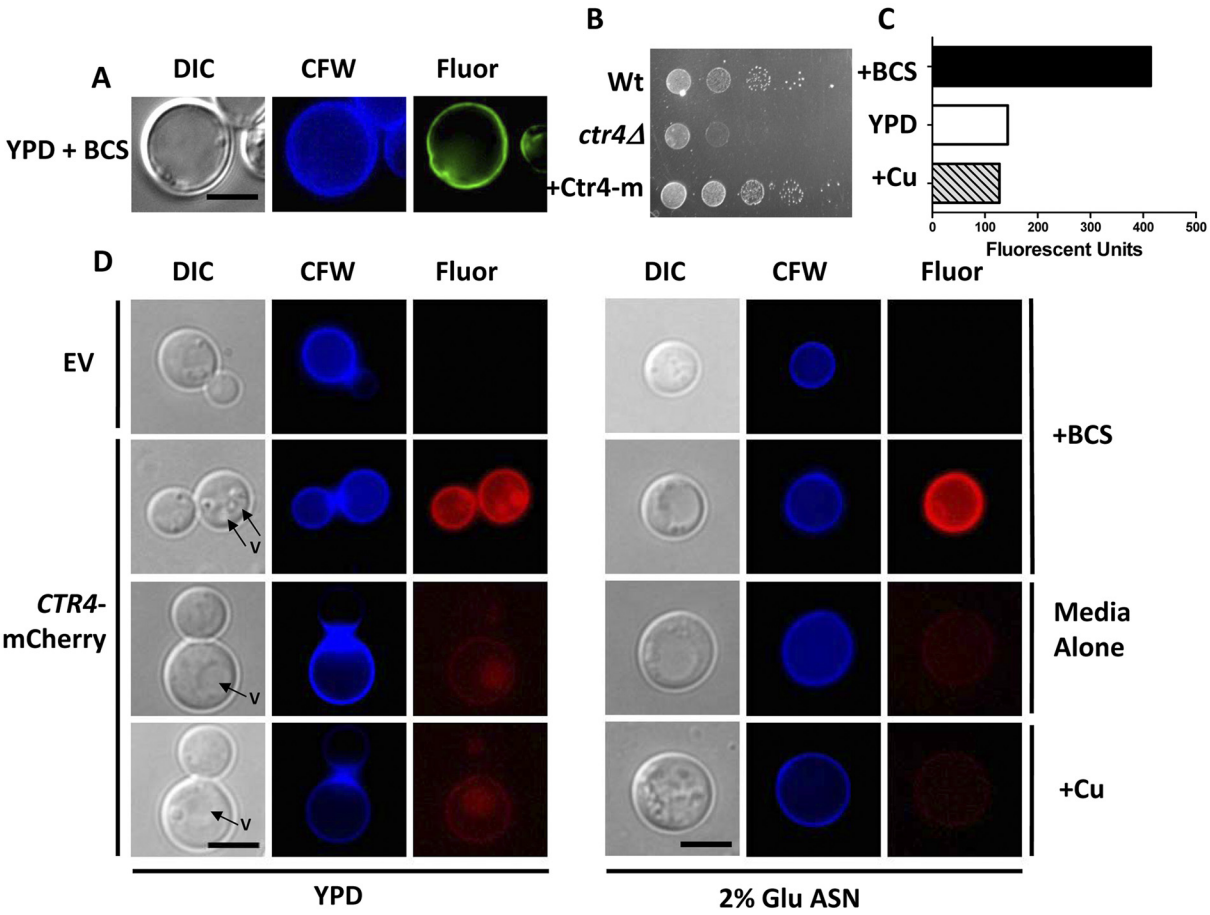

FIG 3 Ctr4 fusion proteins localize to the fungal periphery. Fungal cells were grown in the indicated media (YPD or 2% Glu ASN [2% glucose in asparagine salts]) alone or supplemented with either 100 mM BCS or 30 μM $CuSO_4$ for 2 days at 37°C expressing either a Ctr4-green fluorescent protein (A) or a Ctr4-mCherry fusion protein (B, C, D) and observed by differential interference contrast microscopy (DIC) or fluorescence after labeling with Calcofluor white (CFW). (B) Spot plates of sequential dilutions of indicated strains incubated on 2% glucose-ASN agar and grown at 30°C for 2 days. (C) Expression levels of *C. neoformans* expressing Ctr4-mCherry grown under the indicated conditions at 30°C for 2 days and subjected to flow cytometry. Signal indicates mean fluorescent units of 20,000 cells analyzed (bar = 5 μm). Black arrow points to vacuole (v).

Role of *CTR4* in homeostatic control of copper-related stress. Robust expression of a copper-repressible *CTR4* during infection suggests that specific niches within the host could represent a reduced copper environment. Alternatively, a requirement for *CTR4* under nutrient-limited conditions independent of copper concentration (Fig. 2) could suggest that defective *CTR4*-associated virulence is associated with higher, toxic copper levels as suggested in mycobacterial infections (35). Thus, we used the Δ*ctr4* mutant to probe the role of the gene under higher copper concentrations, first under *in vitro* conditions and then during infection of macrophages and brain. For these experiments, defined medium was first copper reduced by chelation and then supplemented and assayed by inductively coupled plasma mass spectroscopy (ICP-MS) and inoculated with wt and Δ*ctr4* mutant strains. As shown in Fig. 6A, the Δ*ctr4* mutant accumulated a larger copper quota than the wt under all copper conditions, and the copper quota increased for all strains, with increasing copper concentrations up to approximately 10^7 atoms/cell at 30 μM $CuSO_4$. This suggests that at least a subpopulation of copper atoms is extruded from the cell by a *CTR4*-dependent mechanism which was more pronounced at higher copper concentrations. Recovery of cells from brain showed a trend toward higher concentrations of copper in the mutant but was not statistically significant, and the absolute quota (up to approximately 10^7 atoms/cell) corresponded to exogenous copper concentrations in the micromolar range. Attenuation of the copper quota difference between the wt and Δ*ctr4* mutant isolated from the brain could be due to compensatory increases in other copper transporters during infection. To determine if *CTR4* deletion had physiologic consequences at micromolar concentrations of extracellular copper, we next examined expression of the *CMT1* and *CMT2* metallothioneins. Cryptococcal metallothioneins detect elevated levels of intracellular copper and protect cells from copper toxicity (19). As shown in Fig. 6C, Northern blots demonstrated that while *CMT1* and *CMT2* transcription increased in wt cells incubated in 30 μM $CuSO_4$, both metallothioneins showed markedly increased expression in the Δ*ctr4* mutant, suggesting greater cellular stress in the mutant from the increased copper concentration. This suggests that *CTR4* acts in concert with metallothioneins to modulate copper toxicity of the fungus. To provide information on copper cellular stress within the macrophage phagolysosome, a CMT1-GFP reporter strain was constructed which demonstrated observ-

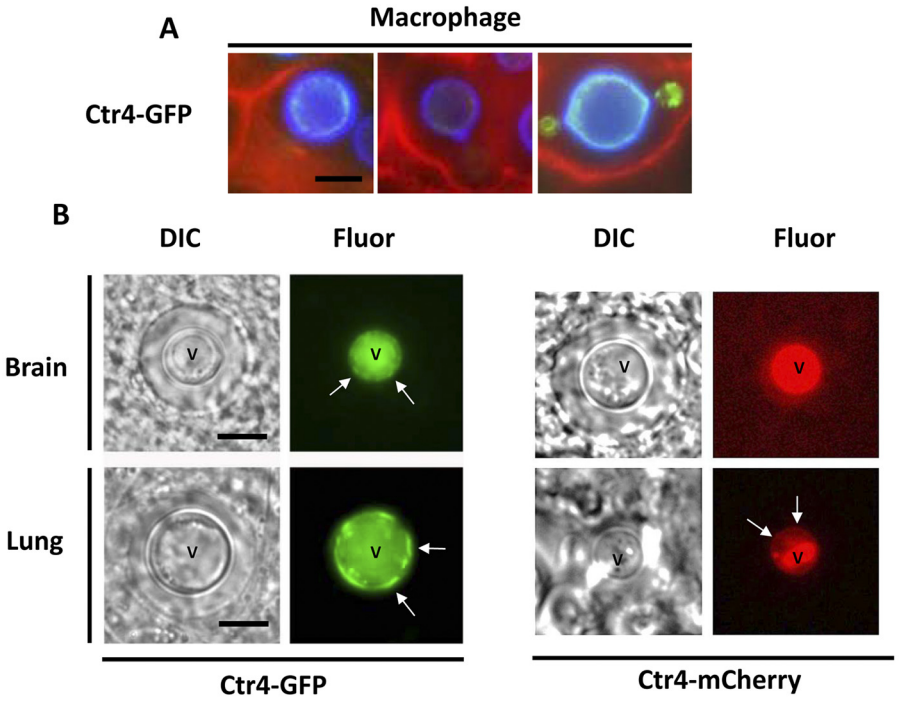

FIG 4 Localization of Ctr4 fusion proteins in macrophages and infected mouse tissue. (A) RAW264.7 cells were infected with fluorescent brightener 28-stained *C. neoformans* expressing Ctr4-GFP and observed by fluorescence microscopy (Fluor) or differential interference microscopy (DIC; bar = 5 μm). (B) Indicated strains were observed within homogenized tissue of i.v. infected mice 9 days postinfection, viewed by fluorescence microscopy (bar = 5 mm, white arrow points to peripherally localized Ctr4, and v indicates yeast vacuole).

able fluorescence under high copper concentrations (1 mM $CuSO_4$). However, uptake of the same strains by RAW264.7 cells, infected as described for Fig. 1B, resulted in no observable fluorescence (Fig. 6D), suggesting that copper concentrations in the phagolysosome were below 1 mM. These data suggest that Ctr4 has a role in homeostatic control of copper at elevated levels and that copper concentrations encountered in the host are likely within the micromolar range.

DISCUSSION

In contrast to iron, the role of copper in microbial pathogenesis has not been extensively explored. While the molecular machinery

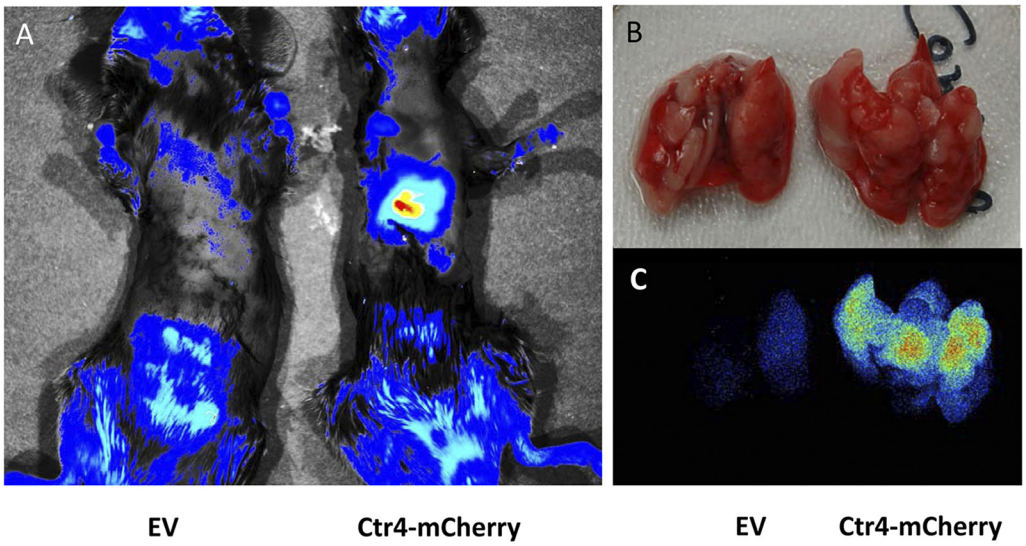

FIG 5 Expression of CTR4-mCherry in lung demonstrated by an *in vivo* optical imaging system. A Δ*ctr4*::Ctr4-mCherry *C. neoformans* strain (Ctr4-mCherry) and a wt *C. neoformans* strain expressing an empty vector (EV) were used to infect C57B/6 mice, and 9 days postinfection, both mice were euthanized. Fluorescence optical imaging of both animals was then performed *ex vivo*. (B) Respective lungs were removed from both animals and reimaged for fluorescent signal (C).

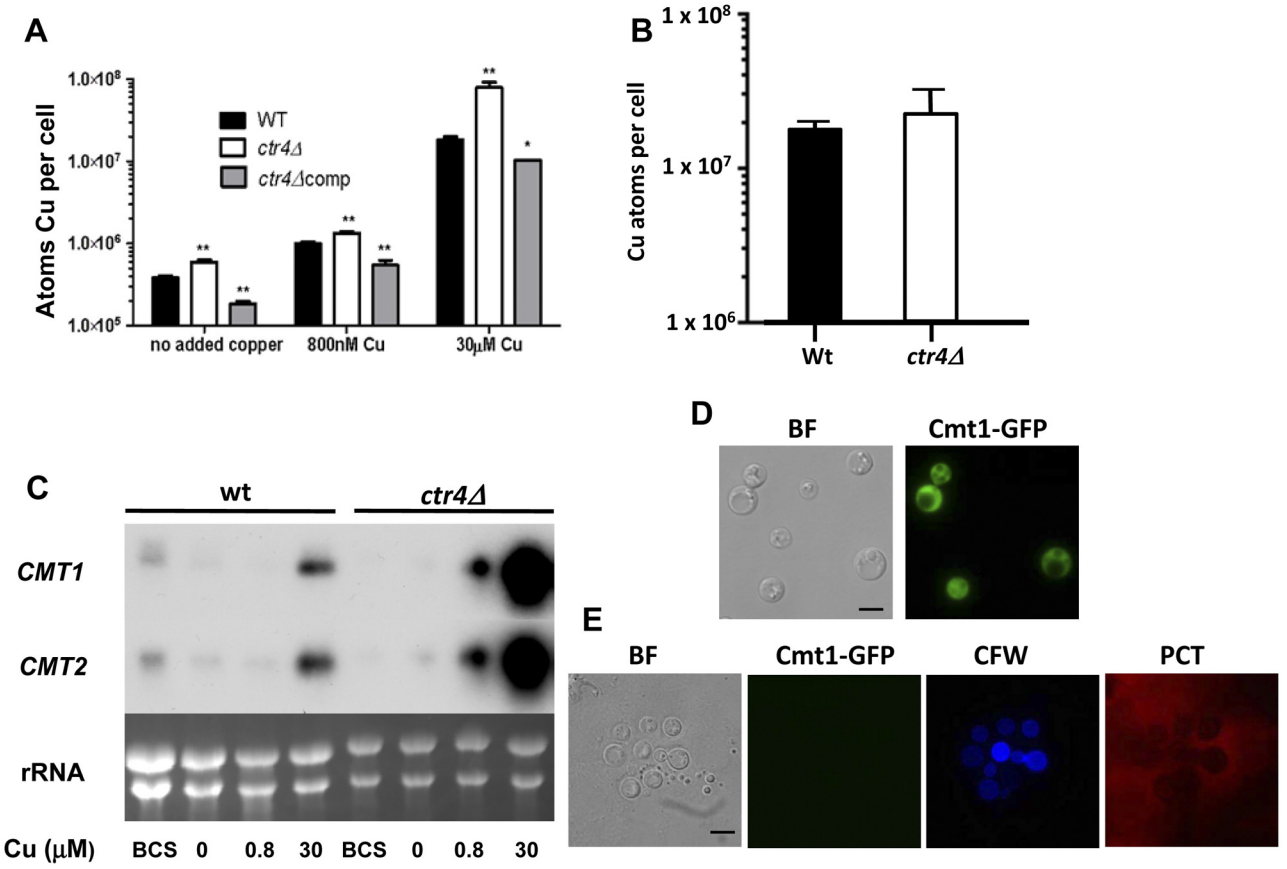

FIG 6 Metallothionein expression studies and copper quota measured by ICP-MS (inductively coupled plasma mass spectroscopy) suggest exposure of *C. neoformans* to micromolar levels of copper during infection. (A) Indicated strains were grown in 2% glucose and YNB containing the indicated concentrations of copper, and copper quota was measured by ICP-MS. (B) Indicated fungal strains recovered from brains of i.v. infected mice and analyzed by ICP-MS. (C) Indicated strains were grown in 2% glucose and YNB, and RNA was recovered and hybridized with ^{32}P-labeled indicated gene fragments. (D) *C. neoformans* cells expressing a Cmt1-GFP fusion protein were grown in 2% glucose and YNB containing 1 mM $CuSO_4$ and viewed by fluorescence microscopy. (E) RAW264.7 cells were infected with Calcofluor-stained (CFW) *C. neoformans* expressing Cmt1-GFP, and macrophages were stained with phalloidin conjugated with Texas Red (PCT) and observed by fluorescence microscopy (bar = 5 μm). BF represents bright field.

involved in copper uptake has been well characterized in nonpathogenic fungi (6), the role of the important copper players has not been elucidated under infectious conditions. The present studies investigated the role of the *CTR4* copper transporter of *C. neoformans* primarily as it relates to virulence in a mammalian host. Ctr4 is one of three copper transporters in *C. neoformans* which has been shown to complement a Δ*ctr1* Δ*ctr3* copper transporter-deficient *S. cerevisiae* mutant (19) and is highly induced under low copper concentrations (18). Previous studies had demonstrated an association between *C. neoformans CTR4* expression in clinical isolates from organ transplant recipients and virulence as measured by a propensity to develop meningoencephalitis versus pulmonary disease (18). However, increased *CTR4* expression in the more virulent clinical strains may have reflected overall activity of the virulence-associated Cuf1 copper transcription factor which regulates a number of stress-related metallothioneins and copper transporters, in addition to *CTR4* (19). Thus, to assess for a direct role for *CTR4* in virulence, a Δ*ctr4* mutant was constructed in *C. neoformans*, which demonstrated defective survival in macrophages as well as attenuated virulence in mice using both an intravenous dissemination model and an intranasal pulmonary model. Subtle alterations in laccase could have contributed to the reduced virulence observed in the mutant strain, although previous studies have not shown strong alterations in virulence with subtle quantitative changes in these virulence factors (29). Seemingly at odds with a role in virulence, previous studies *in vitro* demonstrated that *C. neoformans CTR4* was dispensable for growth under copper-deficient growth conditions, although the authors did report a slight additive growth defect with *CTR1* in a double Δ*ctr1* Δ*ctr4* mutant strain (19). Similarly, the present studies also found no significant growth defect in rich medium (YPD), but further analysis under growth-limiting conditions demonstrated a severe growth defect under more nutrient-restricted conditions in 2% glucose-asparagine salts that was replicated in a second independently transformed mutant and was restored after *CTR4* complementation. Defective growth of the Δ*ctr4* mutant under nutrient-limiting conditions may thus have contributed to its attenuated virulence, as a number of lines of evidence showing the importance of autophagy (31) and high-affinity glucose uptake (36) in virulence suggest the importance of growth/survival under nutrient limitation. Poor survival exhibited in RAW cells may also have been due to this intolerance to starvation, as previous studies showing upregulation of glucose transporters such as Gth1 and Atg9 autophagy-associated

genes after macrophage phagocytosis suggest that the intracellular environment may be relatively nutrient deprived (30). Impaired intracellular survival, in turn, has been linked to reduced virulence in animal models for this facultative intracellular pathogen (3, 37). Use of a murine cell line in the present studies may not fully mimic the intracellular environment of the human macrophage *in vivo*, and further studies using human macrophages may provide interesting insights in the future. Formal clearance studies were not conducted, although the Δ*ctr4* mutant was recovered from brain as described for Fig. 6, suggesting that, despite defective intracellular survival, the mutant was not completely cleared from the host. Interestingly, unlike the mutant of *CTR1* (19), growth of the Δ*ctr4* mutant was not restored by addition of copper. This may be due to a lack of low-affinity copper transporters capable of taking up copper under nutrient-depleted conditions or participation of Ctr4 in a second independent process. Interestingly, in *Schizosaccharomyces pombe*, Ctr4 forms heterocomplexes with the copper transporter Ctr5 to potentiate copper transport (38). In *C. neoformans*, no Ctr5 homolog has been identified, but it is interesting to speculate whether *Ctr4* from *C. neoformans* may form complexes with other biological transporters involved in nutrient transport or synthesis. The possibility of a role in plasma membrane transport is suggested by localization data that found a predominant form of the protein residing in the periphery, consistent with localization in the plasma membrane. However, we were not able to identify a specific amino acid that would restore growth, suggesting that a relationship between Ctr4 and growth under nutrient-limited conditions may be multifactorial and will require further study.

Additional studies of Ctr4 using a newly constructed full-length Ctr4-green fluorescent protein and mCherry fusion protein expression plasmid under the native promoter showed robust expression both in the presence of copper chelators *in vitro* in brain, macrophages, and lung, the latter of which was not evident in previous studies, using a 50-amino-acid truncated Ctr4 protein sequence for the target protein (18). In addition, while full-length Ctr4 fusion protein localized in the periphery as stated above, it also showed accumulation of fluorescent signal in the yeast vacuole as reported previously for the membrane transporter Ctr1 and suggests that Ctr4 protein levels undergo significant protein degradation in *C. neoformans* as reported for the Ctr4 homolog in *S. pombe* (39). More robust expression levels of the *CTR4* fusion proteins and persistence of the fluoroproteins also suggested that *CTR4* expression could be studied in lung using optical imaging, although light attenuation by the skull prevents visualization of fluorescent signal from brain. For lung infection imaging, we used a high-performance system designed for *in vivo* measurement of fluorescence signals in small animals (Maestro 2; CRi, Inc.). These studies showed robust fluorescent signal arising from the lungs of the *CTR4*-mCherry-expressing mouse but not from the lungs of a control mouse having a similar lung fungal burden of an equivalent strain transformed with empty vector alone. Although we performed optical imaging after euthanasia in this study, our findings support the feasibility of *in vivo* optical imaging to detect cryptococcal infections in lung. Development of such a tool may allow an analysis of whole-tissue levels of fungal gene expression and facilitate time course studies of live mouse infections.

While previous expression and complementation studies have suggested a role for Ctr4 under low copper concentrations, we undertook further studies to assess its role under higher copper concentrations since a *CTR4*-dependent growth defect was also exhibited under higher copper concentrations (Fig. 2). Such studies may be helpful in the estimation of copper levels encountered by the fungus during infection. These studies showed that *CTR4* deletion resulted in an increase in cellular copper quota that was exacerbated by higher copper concentrations as measured by ICP-MS. The effect was modest, but increased induction of the copper detoxifier genes *CMT1* and *CMT2* in the Δ*ctr4* mutant under higher copper conditions suggested that this increase was sensed by the fungal cell and was physiologically relevant. Reduced expression of *CMT1* and *CMT2* in wild-type cells also suggests that *CTR4* is synergistic with these factors in homeostatic control of copper toxicity at higher copper concentrations. Cells purified from the homogenized brains of infected mice by sucrose gradients did not show elevations in copper in the mutant strain, which could be due to compensatory efflux from another transporter, but the measured fungal copper quota correlated to studies *in vitro* suggested that the fungus encounters a fairly replete copper environment in the micromolar range during brain infection. Unfortunately, difficulties in the separation of intact fungi from lung tissue due to the increased tissue integrity of lung prevented similar measurements of fungal cells recovered from this organ. Interestingly, recent studies using a hard X-ray microprobe device estimated phagolysosome copper concentrations to be in the micromolar range after uptake of three different species of mycobacteria (40) that was sufficient to inhibit growth of the organism, which displays an unusual copper sensitivity at levels as low as 24 μM (35). In contrast, *C. neoformans* strain H99 is quite copper resistant, displaying normal growth in this same range (Fig. 2) and up to 1 mM (19). Indeed, the present studies showed that *CMT1* was not observed to be induced in *C. neoformans* during macrophage residence where *CTR4* is highly induced, making it less likely that toxic levels of copper in the mM concentrations are encountered during macrophage residence under laboratory conditions. However, unlike *Mycobacteria* and model yeasts, *C. neoformans* is an obligate aerobe, which may require increased copper for respiratory reserve by mitochondrial copper-dependent cytochrome *c* oxidase (12). Thus, increased requirements for copper, rather than copper deprivation *per se*, may be driving *CTR4* expression during infection observed here as well as previously (18). However, estimations of copper concentrations during infection based on genetic data are speculative, as expression levels can be modulated by factors in the host. Estimates of phagolysosomal copper based on intracellular fungal copper quotas should also be interpreted with caution and await direct phagosomal measurements. Nevertheless, these data show the importance for *CTR4* in cryptococcal virulence and its role in copper homeostasis and growth under nutrient-restricted conditions.

MATERIALS AND METHODS

Fungal strains, plasmids, and growth media. The *C. neoformans* ATCC 208821 (H99) strain was a gift from J. Perfect, and H99FOA19 was the recipient strain for the expression of GFP fusion proteins and deletion constructs. The *URA5* gene was contained in pCIP3 (41) and was a generous gift from J. Edman. The nourseothricin resistance gene was contained in pCH233 (42) and was a gift from J. Heitman. Strains were grown in YPD (2% glucose, 1% yeast extract, 2% bactopeptone), YPD agar, or asparagine salts with 2% glucose and 1 g/liter asparagine, 10 mM sodium phosphate (pH 6.5), and 0.25 g/liter $MgSO_4$ as described with additions of SC amino acids (MP Biomedical; MP 4026-032 and MP 4400-022) or a minimal essential medium (MEM) vitamin solution for cell culture di-

luted according to the manufacturer's instructions (Sigma M6895). The RAW264.7 murine macrophage cell line was obtained from ATCC and maintained at 37°C in 5% CO_2 in Dulbecco's modified Eagle's medium (DMEM) (Invitrogen) supplemented with 10% fetal calf serum (FCS).

Disruption and complementation of *CTR4* in *C. neoformans*. Standard methods were used for disruption and complementation as described (13). Briefly, the deletion sequence was constructed by ligating two flanking regions of upstream and downstream *CTR4* genes, generating an internal in-frame deletion of 255 bp. Primers for PCR amplification of the upstream flanking region were CTR4 UpL-BamHI and CTR4-UPR-EcoRINheIPstI (see Table S1 in the supplemental material for primer sequences) and for the downstream flanking region were CTR4 DownL-NheI and CTR4 DownR-NheI. The *URA5* selection marker was PCR amplified by primers URA5 L2-PstI and URA5-PstI; the deletion construct consisted of this fragment flanked by the above-described *CTR4* flanking regions. Each construct generated was confirmed by sequencing. The XbaI/XhoI fragment containing the *CTR4* deletion was gel purified and introduced into H99FOA *C. neoformans* by biolistic transformation. Transformants were selected from 2% glucose in yeast nitrogen base (YNB) and screened by PCR for replacement of the wild-type allele with the disrupted Δ*ctr4* allele. Deletions were confirmed by Southern hybridization, and the absence of *CTR4* gene expression was confirmed by Northern hybridization.

To complement the Δ*ctr4* mutant strain, a 2,967-bp genomic fragment encompassing the full open reading frame (ORF) of *CTR4* plus 1,200 bp of the 5′ promoter region were PCR amplified using a primer set of CTR4 UpL-BamHI and CTR4 ProOrf BamH1R. The PCR product was digested with BamHI and ligated into a modified pBluescript SK vector (Stratagene) containing the 1.6-kb NAT resistance gene under control of a cryptococcal actin promoter. This construct was digested with XbaI/XhoI, gel purified, and introduced into Δ*ctr4* mutant cells by electroporation. Transformants were selected on NAT-containing ASN (2% glucose in asparagine salts [1 g/liter asparagine, 10 mM sodium phosphate {pH 6.5}, and 0.25 g/liter $MgSO_4$] as described previously [22] with or without additions) agar plates (25 μg/ml). Heterologous genomic insertion of the WT *CTR4* and retention of the *URA5* deletion construct in the complement was verified by uncut Southern hybridization and PCR. Expression of the *CTR4* gene was confirmed by Northern hybridization.

Ctr4-GFP and mCherry expression vectors. The Ctr4-GFP fusion protein expression plasmid was constructed by PCR amplifying the *CTR4* promoter and coding region using primers CTR4 ProOrf BamHIL and CTR4 ProOrf EcoRIR. The PCR fragment generated was digested with BamHI/EcoRI and ligated into the BglII/EcoRI sites of a pORA vector described previously (43), and the GFP gene was amplified by PCR with the primers cneoGFPS and cneoGFPA. The resulting construct was confirmed to be in the correct orientation by DNA sequencing and produced a complete Ctr4-GFP fusion protein under the expression of its native promoter. The Cmt1-GFP expression plasmid was constructed by PCR amplifying the *CMT1* promoter region using CMT1 ProL BglII and CMT1 ProR EcoRI and ligating the fragment upstream of the GFP gene of plasmid pORAgfp using compatible restriction sites.

The cryptococcal shuttle vector pORA-KUT was used to express a fusion between the Ctr4 protein and a synthetic mCherry protein (Cneo-mCherry), utilizing *C. neoformans* codon usage produced using standard methods (43) (GenBank accession no. JQ627840). First, pORA-KUT was double digested with BglII and EcoRI, and a PCR-amplified fragment of Cneo-mCherry DNA (obtained using primers RFP-s and RFP-a) was double digested with BglII and EcoRI and then inserted into compatible sites to produce pORA-rKUT. The plasmid was recovered, verified by sequencing, and digested with HpaI, and a PCR-amplified fragment of NAT (obtained using primers NAT-*Hpa* I-s and NAT-*Hpa* I-a) was digested with HpaI and inserted into compatible sites to produce pORA-rKNUT. The plasmid was recovered, the sequence was verified, the plasmid was digested with PstI, and a PCR-amplified fragment of the H99 *CTR4* gene containing the promoter region (obtained using primers CTR4pro-PstI-s and CTR4-PstI-wo ter-a) was digested with PstI and ligated into compatible sites to produce YP164. The plasmids were recovered, the sequences were verified, and the plasmids were linearized with SceI and transformed into *C. neoformans* H99 Matα Δ*ctr4* cells by electroporation using standard methods (33).

Visualization of CTR4-mCherry-expressing *C. neoformans* using optical imaging. Mice were inoculated intravenously with 1×10^6 CFU of either a Δ*ctr4*::CTR4-mCherry *C. neoformans* strain or a wt strain expressing empty plasmid alone and followed until moribund (9 days). Mice were surgically shaved in the thoracic region and subjected to optical imaging using a Maestro 2 *in vivo* imaging system (Cri, Inc., Woburn, MA) with an excitation of 586 to 601 nm, emission of 640 to 820 with 10 nm intervals, and exposure time of 326 ns.

ICP-MS analysis of *C. neoformans*. (i) Preparation of chelexed and reconstituted YNB medium. YNB culture medium was prepared by combining 6.7 g/liter yeast nitrogen base without amino acids (BD, Franklin Lakes, NJ) and 20 g/liter dextrose (Fisher Scientific, Pittsburgh, PA) in ultrapure laboratory-grade water (Millipore, Billerica, MA). Five grams/liter of 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.

ICP-MS was used to measure Mg, Ca, Fe, Ni, Cu, and Zn content in the chelexed and unchelexed YNB medium. The medium was diluted with ultrapure laboratory-grade water (Millipore, Billerica, MA), and 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 90 ppb were made using a mixed element solution (CPI International). Trace metal-grade nitric acid (Fisher Scientific, Pittsburgh, PA) was added to the standards and samples to a final concentration of 2% (wt/vol).

Concentrated stock solutions of $MgSO_4$, $CaCl_2$, $FeCl_3$ $NiSO_4$, $ZnSO_4$, and $CuSO_4$ were measured by weight into ultrapure laboratory-grade water (Millipore, Billerica, MA) and used to reconstitute chelexed YNB to metal levels found in prechelexed medium. Desired copper concentrations were similarly achieved with a concentration solution of $CuSO_4$, also in ultrapure water.

(ii) Culture of *C. neoformans* for ICP-MS. *C. neoformans* strains were grown for 24 h on YPD agar and then inoculated into YPD broth and incubated for 37°C for 16 h. The optical density at 600 nm (OD_{600}) was determined by spectrophotometry, and each culture was washed in ASN medium and resuspended to an OD_{600} of 0.1 in YNB medium containing predetermined concentrations of copper, prepared as described previously. Twenty-five-milliliter cultures were grown in designated YNB medium in Erlenmeyer flasks and incubated at 30°C with shaking at 250 rpm for 48 to 72 h.

(iii) ICP-MS of *Cryptococcus neoformans*. After growth in YNB medium supplemented with the appropriate copper concentration, the cells were transferred to acid-washed Teflon tubes. The cells were washed twice in YNB with 1 mM EDTA and once in ultrapure laboratory-grade water (Millipore, Billerica, MA) with 1 mM EDTA. After the washes, the tubes were uncapped and dried at 70°C overnight. The cells were digested in neat trace metal-grade nitric acid (Fisher Scientific, Pittsburgh, PA) for 30 min at 85°C. The acid solution was diluted with ultrapure 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 90 ppb were made using a mixed element solution (CPI International). The standards and samples all contained 2% (wt/vol) trace metal-grade nitric acid (Fisher Scientific, Pittsburgh, PA). All measurements were performed on an XSeries II ICP-MS instrument (Thermo Scientific, Waltham, MA).

(iv) Isolation of *C. neoformans* from brain tissue. Sets of 10 Swiss albino mice (Harlan) were inoculated intravenously with 10^6 CFU of *C. neoformans*, and the brain tissue was extracted 9 days postinfection (H99). 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 so-

lution (70% [wt/vol]) and centrifuged for 25 min at 3,000 rpm. The *C. neoformans* cell pellets were harvested, washed twice, resuspended in sterile distilled water, and analyzed by ICP-MS. Pilot studies established that copper quotas of fungal cells by ICP-MS did not change significantly during isolation procedures.

Localization of fluorescent fusion proteins by microscopy and quantitation by flow cytometry. Cells were grown under indicated $CuSO_4$ concentration or BCS copper chelator under the indicated conditions and treated with fluorescent brightener (Sigma F3543), 0.01% at 30°C for 10 min. Cells were fixed under a coverslip on a microscope slide and examined for fluorescence, using a Leica DMI 6000B. Expression levels were determined after 2-day growth on the indicated media by flow cytometric analysis using a BD LSRFortessa, excitation 488 nm, emission 505 nm.

Infection of RAW264.7 macrophages with *C. neoformans*. The method used to examine uptake of *C. neoformans* by RAW264.7 cells was as described (44). RAW264.7 cells were grown on coverslips in 24-well Microtek trays and preincubated with IFN-γ for 16 h. To enhance visualization of the *C. neoformans* cells, they were stained with fluorescent brightener 28 (Sigma) for 30 min prior to infection. To enhance phagocytosis of *C. neoformans*, the cells were opsonized with capsular MAb (GMX1 18B7) for 30 min at 37°C prior to infection of cell lines. Following infection, the cells were washed three times with phosphate-buffered saline (PBS) and incubated in DMEM supplemented with FCS (10%) for 48 h. Internalized cells were differentiated from adherent cells using Uvitex 2B (45), which verified in parallel experiments that over 95% of cells were internalized. Coverslips were removed, washed, and stained with phalloidin conjugated with Texas Red for 10 min. Coverslips were placed on slides with mounting medium (Vectashield, Vector Laboratories) and observed under fluorescent deconvolution microscopy.

Duplicate sets of RAW264.7 cells were seeded in triplicate in 24-well plates to a concentration of 10^6 cells/ml. Wells with stimulated cells were inoculated with 5×10^4 CFU of *C. neoformans* cells, centrifuged at 1,000 rpm for 1 min, and allowed to incubate for 2 h. After incubation, the cells were washed 3 times with PBS. One set of wells was lysed with 0.1% Triton (10 min) and vigorous resuspension and plated onto YPD agar. The duplicate set of wells was incubated with DMEM. Twenty-four hours postinfection, the second set of infected cells was washed 3 times with PBS, lysed, and plated. The intracellular replication rate of the *C. neoformans* cells was calculated as a ratio between the 24- and 2-h values.

Virulence factor expression and mouse virulence studies. Capsule formation was assessed using methods previously described (13). Urease production was measured by incubation of cells on Christensen's agar (46), and the method of Liu et al. (47) was used to measure laccase activity. All experimental procedures were conducted under protocols approved by the Institutional Animal Care Committees (IACUC) of the Intramural NIH/NIAID and the University of Illinois at Chicago. For the pulmonary model, CBA/J mice were inoculated intranasally with 1×10^6 CFU of *C. neoformans* wt strains in 20 μl of sterile PBS. The mice were fed *ad libitum* and were monitored by inspection twice daily. Mice were euthanized when moribund. For the intravenous (i.v.) model, sets of 6- to 8-week-old Swiss albino mice (Harlan) were infected by tail vein injection with 10^3 CFU of the indicated strains of *C. neoformans* and mutants. Statistical significance of mouse survival times was assessed by Kruskal-Wallis analysis (analysis of variance [ANOVA] on ranks). Statistical analysis was conducted using GraphPad Prism software, version 4.03.

SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at http://mbio.asm.org/lookup/suppl/doi:10.1128/mBio.00285-12/-/DCSupplemental.

Table S1, DOCX file, 0.1 MB.

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