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Cryptococcus neoformans copper detoxification machinery is critical for fungal virulence

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Summary

Copper (Cu) is an essential metal that is toxic at high concentrations. Thus, pathogens often rely on host Cu for growth, but host cells can hyper-accumulate Cu to exert anti-microbial effects. The human fungal pathogen *Cryptococcus neoformans* encodes various Cu-responsive genes but their role in infection is unclear. We determine that pulmonary *C. neoformans* infection results in Cu-specific induction of genes encoding the Cu-detoxifying metallothionein (Cmt) proteins. Mutant strains lacking *CMT*s or expressing Cmt variants defective in Cu-coordination exhibit severely attenuated virulence and reduced pulmonary colonization. Consistent with the up-regulation of Cmt proteins, *C. neoformans* pulmonary infection results in increased serum Cu concentrations and respectively increases and decreases alveolar macrophage expression of the Cu importer, Ctr1, and ATP7A, a transporter implicated in phagosomal Cu compartmentalization. These studies indicate that the host mobilizes Cu as an innate anti-fungal defense but that *C. neoformans* senses and neutralizes toxic Cu to promote infection.

Introduction

Copper (Cu) has a long history as an anti-microbial agent, employed to sterilize wounds by the ancient Egyptians, to ward off cholera in the 19th century, and as an anti-fungal agent in Bordeaux mixture in vineyards (Cassat and Skaar, 2012; Hodgkinson and Petris, 2012; Hood and Skaar, 2012; Samanovic et al., 2012). More recently, Cu surfaces are utilized in healthcare settings to reduce nosocomial infections (Schmidt et al., 2012). While the precise mechanisms by which Cu exerts anti-microbial activity are not well understood, the redox properties of this metal foster the generation of toxic hydroxyl radicals (•OH) and hydroxyl anions (OH^-), which can cause DNA and protein damage (Halliwell and Gutteridge, 1985).

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Furthermore, Cu hyper-accumulation has been shown to interfere with Fe-S clusters that are critical to enzymes involved in a plethora of essential biochemical processes (Chillappagari et al., 2010; Liochev, 1996; Macomber and Imlay, 2009; Macomber et al., 2007).

The phagosomal compartment of innate immune cells presents a hostile environment to invading microbial pathogens via the generation of reactive oxygen and nitrogen species, the elaboration of proteases and other degradative enzymes, acidification of the phagosomal lumen and by nutritional limitation of metals such as Fe, Zn and Mn that are essential for microbial growth (Hood and Skaar, 2012; Nathan and Shiloh, 2000). While phagocytic cells sequester these metals from invading pathogens, macrophages infected with *Mycobacterium* species hyper-accumulate Cu within the phagosome (Wagner et al., 2005). Moreover, macrophage cell lines that have been activated with IFN-γ elevate expression of both the plasma membrane Cu^+ importer, Ctr1, and the ATP7A vesicular Cu pump (White et al., 2009). As ATP7A is thought to traffic to the phagosomal membrane in these cells, and ATP7A depletion enhances *E. coli* survival to macrophage killing, these observations suggest that elevated luminal Cu is microbiocidal (White et al., 2009).

Cryptococcus species such as *C. neoformans* are pathogenic fungi that cause cryptococcosis in both immunodeficient and immunocompetent individuals. *C. neoformans* is acquired from the environment through inhalation, disseminates through the bloodstream to the brain and causes ~600,000 deaths annually from lethal meningitis (Heitman, 2011; Kronstad et al., 2012; Kronstad et al., 2011). Previous studies demonstrated that the metals Fe and Cu play important roles in *C. neoformans* virulence, because they are directly involved in many key biochemical processes (Jung et al., 2009; Jung et al., 2008; Jung et al., 2006; Salas et al., 1996; Walton et al., 2005; Williamson, 1994). In particular, Fe is critical for heme biosynthesis, oxidative phosphorylation and serves as a critical cofactor for dozens of enzymatic reactions. Cu functions in melanin formation, Fe uptake, reactive oxygen detoxification and respiration (Ding et al., 2011; Jung et al., 2009; Jung et al., 2008; Jung et al., 2006; Kronstad et al., 2012; Samanovic et al., 2012; Williamson, 1994). Melanin, a protective pigment and virulence factor, is synthesized by *C. neoformans* via the secreted Cu-dependent oxidase laccase, using host brain catecholamines as substrate (Williamson, 1994). Accordingly, deletion of the genes encoding laccase, or the secretory compartment Cu importer Ccc2, severely compromised *C. neoformans* virulence (Salas et al., 1996; Walton et al., 2005). The *C. neoformans* Cu metalloregulatory transcription factor Cuf1 has also been demonstrated to be important for virulence (Waterman et al., 2007). Since Cuf1 plays a critical role in activating expression of the *CTR4* gene, encoding a high affinity plasma membrane Cu^+ importer, Cu acquisition was proposed to underlie the requirement for Cuf1 for virulence (Waterman et al., 2007). However, additional studies demonstrated that *cuf1*Δ mutants exhibit both Cu sensitivity phenotypes and growth defects under Cu deficient conditions (Ding et al., 2011; Lin et al., 2006). Accordingly, we demonstrated that Cuf1 activates the transcription of genes encoding the Cu acquisition machinery (*CTR1* and *CTR4*), or genes encoding the Cu detoxification machinery (*CMT1* and *CMT2*), under Cu limitation or Cu excess, respectively (Ding et al., 2011). Given the role of Cuf1 target genes in both Cu acquisition and detoxification, it is important to clarify the specific functions of the Cuf1 regulon in virulence.

Here we report that live animal imaging studies using specific Cu-activated reporters responsive to either high Cu, or Cu deficiency, demonstrate that *C. neoformans* high Cu-induced reporter is dramatically induced during initial respiratory colonization. We demonstrate that the *C. neoformans* metallothioneins, which are induced in a Cu-specific manner and have a high capacity for Cu binding, play a critical role in virulence. Analysis of host Cu homeostasis proteins in bronchoalveolar lavage (BAL) cells from infected animals showed a dramatic increase in the high affinity mammalian Cu importer, Ctr1, and

decreased abundance of the ATP7A Cu transporter that has been implicated in phagosomal Cu compartmentalization.

Results

C. neoformans metallothionein gene expression is activated in lung infection

We previously demonstrated that *C. neoformans* genes encoding metallothioneins or Cu transporters are strongly induced under high or low Cu conditions, respectively, in a Cu concentration dependent manner (Ding et al., 2011). Here, using qRT-PCR as a sensitive and quantitative assay, Cmt1 mRNA levels were induced ~800 fold in response to Cu and Ctr4 mRNA levels were induced ~600 fold in response to the Cu^{+}-specific chelator BCS (Figure 1A). To ascertain whether these genes are directly regulated by Cuf1 in response to Cu levels, a FLAG-epitope tagged Cuf1 allele was generated and expressed in *cuf1*Δ cells for use in Chromatin immunoprecipitation (ChIP) experiments. Cuf1 was tagged with 2 copies of the FLAG sequence at the carboxyl-terminus, and *cuf1*Δ strains transformed with this expression plasmid are fully complemented with respect to the Cu and BCS sensitive phenotype of *cuf1*Δ cells, demonstrating that this is a functional Cuf1-Flag protein (Figure S1A). ChIP assays followed by qPCR analysis of promoter sequences from the *CMT1/2* and *CTR1/4* genes showed strong Cu-regulated Cuf1 binding to the *CTR1* and *CTR4* promoters under Cu deficiency as compared to high Cu conditions. In contrast, Cuf1 binding to the *CMT1* promoter was induced under high Cu conditions, with binding to *CMT2* observed under both conditions (Figure 1B). These results demonstrate that Cuf1 plays a direct role in the activation of Cu detoxification genes and Cu acquisition genes, when cells encounter distinct Cu environments.

To assess the potential Cu environment in host tissue sensed in the initial stages of *C. neoformans* infection through its natural respiratory route of infection, two Cu-responsive reporter plasmids were constructed in which luciferase expression is driven by the *C. neoformans CTR4* promoter (*CTR4*-Luc) in response to Cu limiting conditions or the *CMT1* promoter (*CMT1*-Luc) in response to elevated Cu. *CMT1*- and *CTR4*- driven luciferase protein expression and activities from each reporter was confirmed by luciferase enzyme assays and immunoblotting (Figure 1C and S1B). In *C. neoformans* cells luciferase activity from the *CMT1* promoter is induced 17-fold in response to increasing Cu, while activity from the *CTR4* promoter is induced 14-fold in response to BCS treatment; no activity was detected from cells lacking a luciferase reporter (Figure 1C). Intranasal infection of mice was carried out with independent isolates of *C. neoformans* carrying an integrated copy of the *CTR4*-Luc or *CMT1*-Luc reporter, followed by live animal imaging and luciferase activity quantitation (Figure 1D and E). After 2 days weak activity was detected in animals infected with cells harboring the *CMT1* or *CTR4* reporters, but not with control cells. While luciferase activity for the *CTR4*-luc infection remained low and unchanged throughout the subsequent 14 day infection period, there was a time-dependent increase in *CMT1*-driven luciferase activity in lung tissue. To ascertain whether the difference in luciferase activity between the two reporter strains is due to impaired lung colonization by cells harboring the *CTR4*-Luciferase reporter, we performed fungal burden assays and detected no difference in lung fungal cell burden between the two reporter strains (Figure 1F). These results suggest that the Cmt1 gene is induced, when *C. neoformans* is acquired by the respiratory route, the natural route of infection in humans.

C. neoformans MTs are critical factors for lung colonization and virulence

Expression from the *C. neoformans CMT1* promoter is activated in pulmonary infection, implying that fungal cells sense elevated Cu in the lung, and Cuf1 directly activates transcription of the *CMT1* and *CMT2* genes, whose encoded proteins are required for Cu

detoxification in *C. neoformans* (Figure 1). Consequently, the potential role of *CMT1* and *CMT2* in *C. neoformans* virulence was investigated by infecting mice with wild type or isogenic *cmt1*Δ, *cmt2*Δ or *cmt1*Δ *cmt2*Δ mutants (Figure 2A). While *CMT1* and *CMT2* are functionally redundant for Cu detoxification (Ding et al., 2011), an *cmt1*Δ *cmt2*Δ mutant is over 30-fold more Cu-sensitive than WT cells *in vitro* (IC_{50} for WT of 2.3 mM vs. 73 μM for *cmt1*Δ *cmt2*Δ). Deletion of either *CMT1* or *CMT2* did not alter mouse survival compared to the parental strain. However, the *cmt1*Δ *cmt2*Δ strain was strongly attenuated in virulence (Figure 2A). Two independently generated *cmt1*Δ *cmt2*Δ strains were evaluated for lung tissue burden 14 days post infection, with both *cmt1*Δ *cmt2*Δ strains showing a dramatic decrease in lung tissue fungal burden in comparison to the wild type strain (Figure 2B). This observation was validated by staining tissue sections for *C. neoformans* with the capsule-specific stain mucicarmine, which showed a reduction in *cmt1*Δ *cmt2*Δ cells as compared to wild type cells, with no defect in melanin production, capsule formation or phagocytosis observed between wild type and *cmt1*Δ *cmt2*Δ cells (Figure S2). Furthermore, a corresponding decrease in host lung tissue damage was evident as determined by H&E staining of lung tissue sections (Figure 2C). Taken together, these results are correlate with the strong expression of *CMT1* in lung observed in live animal imaging studies, and demonstrate that *C. neoformans* Cmts are required for full fungal virulence when acquired via the respiratory route, the natural route of infection.

C. neoformans MTs are atypical metallothioneins specifically activated by Cu

MTs are expressed in organisms from prokaryotes to humans, which bind metals through Cys thiolate bonds (Butt et al., 1984; Kagi and Hunziker, 1989; Szczypka and Thiele, 1989; Winge and Nielson, 1984; Winge et al., 1985). We previously reported that Cmts from *C. neoformans* possess Cu binding motifs typical of MTs (CxC), yet they are atypical MTs compared with those from other species (Ding et al., 2011). Both Cmt1 and Cmt2 are much larger proteins, where Cmt1 is 122 amino acids and Cmt2 is 183 amino acids long as compared to human MT1A of 61 amino acids. Multiple sequence alignments demonstrated that metallothioneins from *Cryptococcus* share homology with the MTs from *S. cereviaise* and humans (Figure S3). Phylogenetic analysis suggests that Cmt1 and Cmt2 share the same evolutionary origin, but are distantly related to the Crs5 and Cup1 MTs from *Saccharomyces cerevisiae* and human MT1A and MT2A (Figure 3A). Comparison of protein sequences between *C. neoformans* MT1 and MT2 reveals that both proteins are divided into multiple Cys-rich sequence segments by spacer sequences termed B1 to B4 (Figure 3B), with Cmt1 divided into three segments by three spacer regions and Cmt2 harboring three Cys segments separated by four spacer regions. The spacer regions between Cmt1 and Cmt2 share high similarity for B1 and are identical for B2 and B3. Interestingly, the Cys-rich motif resembles that found in MTs from other fungi such as *Agaricus* and *Neurospora* and may imply evolutionary divergence from a common ancestor among these species (Figure 3C).

Mammalian MT genes are transcriptionally induced by metals that include Zn, Cd, Cu and Ag and protect cells against these and other metals (Durnam and Palmiter, 1984, 1987; Kagi and Hunziker, 1989). To decipher the specificity of metal detoxification with respect to *Cryptococcus* MTs, we measured cell growth in liquid medium supplemented with a range of metal concentrations including Cu, Zn, and Cd. The effect of high and low Fe on cell growth was also tested because Fe acquisition via the Fe permease is directly dependent on a multi-Cu oxidase in *C. neoformans* (Jung and Kronstad, 2008; Kronstad et al., 2012). A potential role for the *C. neoformans* MTs for growth in the presence of reactive oxygen species was also tested using the superoxide generator, menadione. We observed a striking growth defect of *cmt1*Δ *cmt2*Δ cells in the presence of Cu, with no significant difference in the presence of Cd, Zn, Fe, the Fe chelator BPS or the superoxide generator menadione (Figure 4A). Agar spotting assays were also performed to confirm the liquid growth

experiments, and similar cell growth phenotypes were observed (Figure 4B). Consequently, we determined whether RNA and protein expression of *C. neoformans* MTs are elevated in response to these conditions by qRT-PCR and immunoblot assays. As shown in Figures 4C and 4D, expression of both *CMT1* and *CMT2* mRNA and FLAG-epitope-tagged protein is strongly elevated in response to Cu exposure in a dose-dependent manner, but not in response to any concentration of Zn, Fe, Cd, BPS or Menadione tested. Taken together, these results demonstrate that, of all conditions tested, the *C. neoformans* MT genes are Cu responsive and function specifically in Cu detoxification.

To evaluate the importance of Cu binding by Cmt1 and Cmt2 to Cu detoxification, the importance of the Cmt1 Cys residues in protecting cells from Cu toxicity was tested. A DNA sequence encoding a *CMT1* allele in which all Cys residues were converted to Ala was synthesized, cloned under control of the *CMT1* promoter and introduced into *cmt1*Δ *cmt2*Δ cells to generate the Cmt1ala strain. Using qRT-PCR, the expression of *CMT1ala* was confirmed to be robust and Cu responsive, as the fold induction of expression between Cu and BCS treatment is comparable to that observed for wild type *CMT1* (Figure S4A). Cell growth assays demonstrated that the Cmt1 Cu-coordinating Cys residues are essential for Cu resistance (Figure 5A).

To quantify the Cu binding capacity of Cmt1 and Cmt2, the Cmts were synthesized in and purified from *E. coli*, after which spectroscopic analysis of Cmt1, Cmt2 and Cmt1ala was performed. These experiments showed a high, preferential Cu^+ binding capacity yielding major homonuclear Cu_{16}-Cmt1 and Cu_{24}-Cmt2 complexes (Figure 5B and C). *In vitro* Zn/Cu replacement experiments using recombinantly synthesized Zn-Cmt1 and Zn-Cmt2 complexes fully corroborated these stoichiometries (Figure S4B and C) and pointed to the progressive and cooperative formation of several Cu_5 ion clusters (3 for Cmt1 and 5 for Cmt2), in accordance with the peculiar protein architecture in the same number of Cys-rich regions (Figure 3). Furthermore, the CD spectra of the complexes and the products of recombinant synthesis were nearly identical (Figure S4D and E), indicating equivalent folding. Consistent with the inability of the Cmt1ala mutant to support Cu-resistance in *cmt1*Δ *cmt2*Δ cells (Figure 5A), the Cmt1ala protein was defective in Cu^+ binding and was isolated exclusively in the apo form (Figure 5D). These results establish Cu^+ binding to the *C. neoformans* MTs with high stoichiometry that is dependent on Cys thiolate bonds. To test whether the cysteine residues of *CMT1* are required for virulence, mice were infected with wild type *C. neoformans* cells, isogenic *cmt1*Δ *cmt2*Δ cells, or the same mutant strain expressing either *CMT1*, *CMT2* or *CMT1ala* and fungal burdens were evaluated in host lung tissue. Consistent with the Cu binding results, *C. neoformans* expressing a Cmt1 protein that is incompetent for Cu binding (Cmt1ala) exhibited poor survival as evidenced by decreased fungal burden in lung tissue from infected mice (Figure 5E). These results demonstrate the essential role of the Cmt1 Cys residues, required for Cu^+ coordination, for virulence in mouse lung infection.

Although MTs have been previously localized to the cytosol of fungal and mammalian cells (Banerjee et al., 1982; Hamer, 1986; Winge and Nielson, 1984), the subcellular distribution of Cmt1 and Cmt2 was determined in cells cultured *in vitro*. The expression of functional FLAG-epitope tagged Cmt1 and Cmt2 (Figure 6A) was confirmed by immunoblotting experiments in which an ~20 KDa polypeptide was detected for Cmt1-FLAG and ~37 KDa species for Cmt2-FLAG (Figure 6B. Subcellular localization experiments by indirect immunofluorescence microscopy of Cu-treated *C. neoformans* cell cultures demonstrated that Cmt1-FLAG and Cmt2-FLAG concentrate at the cell periphery (Figure 6C). This observation was recapitulated by immunohistochemistry analysis of lung tissue infected with *C. neoformans* cells expressing either Cmt1-FLAG or Cmt2-FLAG (Figure 6D). Taken together, these experiments demonstrate that a Cu-binding Cmts is critical for both *C.*

neoformans Cu resistance *in vitro* and virulence in mouse infections. Furthermore, distinct from the pancellular localization of Cmts observed in other eukaryotes (Hamer, 1986), the *C. neoformans* atypical MT proteins concentrate at the cellular periphery.

C. neoformans infection alters host Cu mobilization and Cu transporter expression

Bronchial alveolar macrophages are phagocytic cells that provide the first line of defense against *C. neoformans* infection in the lung (Brummer, 1998; Kronstad et al., 2011). Previous *in vitro* studies demonstrated that macrophage-like cell lines infected with *Mycobacterium* spp. accumulate Cu in the phagosomal compartment and activation of macrophage cell lines with LPS induces expression of the ATP7A Cu^+-transporting P-type ATPase and the Ctr1 high affinity Cu^+ importer (Wagner et al., 2005; White et al., 2009). Macrophage cell lines with reduced expression of ATP7A are deficient in killing *E. coli*, consistent with a potential role for ATP7A in phagosomal microbiocidal Cu^+ loading (White et al., 2009). Because activated macrophages have elevated ATP7A and Ctr1 levels, and we show here that *C. neoformans* senses high Cu and activates the *MT1* promoter during lung infection, we ascertained whether there are changes in host circulating Cu levels and in expression of the host Cu homeostatic machinery in response to *C. neoformans* infection. Serum Cu levels were significantly increased, suggesting a mobilization of host Cu in response to *C. neoformans* infection (Figure 7A). Moreover, cells from mouse bronchoalveolar lavage (BAL) 14 days after infection, of which the dominant cell type has been shown to be alveolar macrophages (Giles et al., 2007), displayed a strong decrease in the steady-state levels of ATP7A (Figure 7B) that was observed to a lesser extent 2 days post-infection (Figure 7C). In contrast, infected mice exhibited no apparent change in lung tissue ATP7A levels compared to uninfected controls (Figure 7D). After 14 days of infection the levels of the Ctr1 high affinity Cu^+ importer, and the Cox IV subunit of mitochondrial cytochrome oxidase whose levels correlate with intracellular Cu availability, were strongly elevated (Figure 7B). Taken together, these observations suggest that in response to *C. neoformans* infection via the respiratory route, host mobilize Cu into the circulation and lung alveolar cells may re-orient Cu away from vesicular compartments and toward the mitochondria or other pools.

Discussion

Prokaryotic Cu detoxification mechanisms involving Cu-responsive transcription factors and Cu efflux pumps are emerging as critical virulence factors for organisms such as *M. tuberculosis*, *E. coli*, *S. enterica* and others (Achard et al., 2010; Osman and Cavet, 2011; Samanovic et al., 2012; Schwan et al., 2005; Wagner et al., 2005; White et al., 2009; Wolschendorf et al., 2011). In line with these observations are studies that demonstrate the compartmentalization of Cu within the macrophage phagosome in response to infection (Wagner et al., 2005; White et al., 2009), in a manner that correlates with elevated expression of the mammalian Ctr1 plasma membrane Cu^+ importer and the ATP7A Cu^+ transporting ATPase (White et al., 2009). As depletion of ATP7A renders macrophages more permissive for *E. coli* survival (White et al., 2009), and mice receiving dietary Cu supplements more effectively clear *M. tuberculosis* (Wolschendorf et al., 2011), these and other experimental results point to phagosomal Cu compartmentalization, that may involve ATP7A, as a potent anti-microbial weapon against infectious disease (Hodgkinson and Petris, 2012; Rowland and Niederweis, 2012; Samanovic et al., 2012; Wolschendorf et al., 2011).

Pathogenic fungi such as *C. neoformans* and *C. albicans* are quite resistant to Cu levels *in vitro* (Ding et al., 2011; Weissman et al., 2000), with *C. neoformans* H99 resistant to ~2 mM Cu in liquid medium and clinical isolates of *C. albicans* able to tolerate ~20 mM Cu (Weissman et al., 2000). An important question is why *C. neoformans*, or other pathogenic

fungi, are tolerant to such high Cu concentrations and is this relevant to the concentrations of Cu they encounter during infection? In contrast to Cu detoxification in prokaryotes, Cu acquisition has been implicated in virulence by *C. neoformans* in mouse infection models (Waterman et al., 2007). Cuf1 was previously implicated in virulence by mouse tail-vein administration studies, and its known activation of *CTR4* implied a requirement for Cu for virulence (Waterman et al., 2007). More recent studies using *URA5* to disrupt *CTR4* resulted in *C. neoformans* cells with a pleiotropic nutritional deficiency that was not corrected by external Cu and resulted in a reduction in virulence in mice (Waterman et al., 2012). However, given that such a growth phenotype has not been observed in Cu transporter knockouts of Ctr1 or Ctr4 in *C. neoformans* by us (Ding et al., 2011), or in response to inactivation of other Cu importer genes in *S. cerevisiae*, *S. pombe* or *C. albicans* in other studies (Beaudoin et al., 2006; Dancis et al., 1994; Marvin et al., 2003; Pena et al., 2000; Zhou and Thiele, 2001), it is not clear why this pleiotropic phenotype was observed. One possibility is that use of the *URA5* marker for gene disruption in *C. neoformans*, and the *URA3* marker in *C. albicans* and *C. parapsilosis*, has been shown to cause defects in adhesion, colony morphology and virulence that are unrelated to the target genes of interest (Bain et al., 2001; Ding and Butler, 2007; Kirsch and Whitney, 1991; Kwon-Chung et al., 1992; Lay et al., 1998; Staab and Sundstrom, 2003). While it is possible that the Cu acquisition machinery may contribute to the colonization of lung and brain due to a requirement to activate Cu, Zn SOD (Bermingham-McDonogh et al., 1988; Furukawa et al., 2004), the Cu dependent oxidase involved in Fe uptake (Dancis et al., 1994; Jung and Kronstad, 2008), and for the synthesis of melanin from laccase (Walton et al., 2005; Williamson, 1994) using host catecholamine as substrate, the reasons behind these discrepant studies merits further investigation.

A recent study using a *CTR4*-Cherry reporter suggested that *CTR4* is strongly expressed in macrophages *in vitro* and in lung and brain tissue (Waterman et al., 2012). However, *CTR4*-driven expression of mCherry in this study was compared to that of *C. neoformans* cells harboring an empty vector without the mCherry gene. In this work we used *C. neoformans* cells harboring high and low Cu-responsive reporter plasmids, as well as negative control cells, to quantitatively ascertain, over the course of a 14-day intranasal infection, whether *C. neoformans* is exposed to a high or low Cu environment. While both *CTR4*-luc and *CMT1*-luc are expressed in lung during the initial phase of the infection, the *CMT1*-luc reporter was activated in a time-dependent manner while the *CTR4*-luc remained low and constant. The strong induction of the *CMT1*-luc reporter in lung implies that *C. neoformans* senses elevated Cu levels in the lung. Consistent with this observation, we demonstrated that the *CMT1* and *CMT2*, and a Cu-binding competent Cmt1 protein, are required for virulence at the natural site of acquisition, the lungs. Indeed, in contrast to mammalian MTs, the *C. neoformans* MTs, and other fungal MTs, are transcriptionally activated in response to Cu, rather than to any other metal tested, suggesting a role that is specific under conditions of high Cu. Moreover, we show that the *C. neoformans* MTs are longer and have an exceptionally high Cu binding capacity compared to other MT proteins, perhaps due to evolutionary pressure to evolve by tandem amplification of a basic Cu binding unit similar to that found in well characterized fungal metallothioneins. The concentration of Cmt1 and Cmt2 to the cell periphery, via currently uncharacterized targeting mechanisms, could provide a means to efficiently capture Cu^{+} immediately after it enters cells, prior to engaging in redox chemistry, interfering with Fe-S clusters or targeting other mechanisms for toxicity (Chillappagari et al., 2010; Liochev, 1996; Macomber and Imlay, 2009; Macomber et al., 2007).

The results presented here showing a requirement for *CMT1* and *CMT2* for virulence are consistent with macrophages in the lung and other tissues using Cu as an antimicrobial condition within the lumen of the phagosome. As the expression of ATP7A and Ctr1 was

shown to be elevated in activated macrophage cell lines, and a fraction of ATP7A was found in the phagosomal membrane, this Cu^+ pump is implicated as a potential driver of phagosomal compartmentalization of anti-microbial Cu (White et al., 2009). Complementary to the elevation of ATP7A in INF-γ activated macrophages *in vitro*, we found that *C. neoformans* infection caused a time-dependent down regulation of ATP7A in lung lavage cells, an environment reported to be composed predominantly of phagocytic cells (Giles et al., 2007). In addition to the Cuf1-dependent Cu detoxification genes, this could provide a survival advantage to *C. neoformans* within the phagosomal compartment that ultimately allows this organism to escape into the cytoplasm by vomocytosis (Nicola et al., 2011). While the mechanism for ATP7A dampening, but maintenance of high Ctr1 levels, is currently unknown, *C. neoformans* infection is known to cause a reduction in host pro-inflammatory cytokines and an increase in NF-κB activity, via glucuronoxylomannan in the outer capsule (Ben-Abdallah et al., 2012; Piccioni et al., 2013), which may reduce ATP7A expression. We speculate that as the Ctr1 promoter, but not that of ATP7A, contains a putative NF-κB binding site (http://genome.ucsc.edu/ENCODE) (Dunham et al., 2012), this could maintain Ctr1 expression while ATP7A levels are tuned down. It is likely that the regulation of Ctr1 and ATP7A expression is due to a complex interplay between *C. neoformans* and the host immune system and this should be explored in more detail. The increased levels of circulating Cu and the high levels of Ctr1 and Cox IV in the host could suggest that *C. neoformans* infection results in the down-regulation of the host Cu compartmentalization machinery, potentially reorienting available Cu to other intracellular targets such as the mitochondria. The use of *C. neoformans* mutants, in concert with mouse models with altered Cu homeostasis, could help elucidate the detailed mechanisms by which Cu functions at the host-pathogen axis.

Experimental Procedures

Strains and media

Cryptococcus neoformans H99 strains (Table S1) were routinely grown as previously described (Ding et al., 2011). YPD agar supplemented with 100 mg/L G418 or 200 U/ml hygromycin B was used for colony selection. Mutants were generated as described in Supplemental procedures.

Chromatin Immunoprecipitation

Cells expressing Cuf1-FLAG were treated with 1 mM Cu or BCS for 3 hrs. Cell fixation and ChIP were performed as described previously (Pondugula et al., 2009), except buffer (50 mM HEPES, 140 mM NaCl, 1% Triton X-100, 1 mM EDTA, protease inhibitors) was used to lyse cells and M2 beads (Sigma) were used for immunoprecipitation. Promoter sequences from *CMT1, CMT2, CTR1, CTR4* and *TUB2* was analyzed using qPCR. Primer sequences are described in the Supplemental procedures.

Luciferase assays and live animal imaging

Strains transformed with luciferase reporter genes were diluted to an A_{600} of 0.2 in SC medium supplemented with Cu or BCS and incubated at 37°C for 9 hrs. Cell cultures were washed and resuspended in PBS.10 μl of cell suspension was mixed with 100 μl with luciferase reporter reagent (Promega) and activity was measured using a bioilluminator Victor (PerkinElmer). The samples were then measured at A_{600} for cell number.

A/J mice were infected with wildtype, *CTR4*-Luc, or *CMT1*-Luc strains intranasally. Mice were anesthetized using 2.5% of isoflurane. Luciferin was introduced intranasally into each animal (no signal was observed when luciferin was administered intraperitoneally). Animals were placed in a Caliper IVIS Spectrum (PerkinElmer) chamber at 37°C. The scan was

performed exactly 5 min after introducing luciferin. Scanning was performed at day 0, 2, 7, 9, 12 and 14. Animals were sacrificed at day 14 for CFU analysis. All images were analyzed using Living Image 4.2 (Caliper, PerkinElmer). The lung region from each animal was cropped and total luciferase signal intensity in the cropped area was extracted using Living Image 4.2. Statistical analysis was performed using the *student t-test*.

Animal infection, fungal burden assay and histopathology

Animal infections were performed as described previously (Crabtree et al., 2012). Histology of uninfected or infected lung tissue was processed and mucicarmine or hematoxylin/eosin staining was performed.

Spectroscopic analyses and Electrospray Ionization Mass Spectrometry (ESI-MS)

Cmt proteins were expressed in *E. coli BL21* strain and purified using GST fusion as described in the Supplemental procedures. The S, Zn and Cu content of the Zn- and Cu-Cmt preparations was analysed by Inductively Coupled Plasma Atomic Emission Spectroscopy (ICP-AES) as described previously (Bongers et al., 1988; Capdevila et al., 2005).

Molecular weight determinations were performed by electrospray ionization time-of-flight mass spectrometry (ESI-TOF MS). The calibration was attained with 0.2 g NaI dissolved in 100 ml of a 1: H_2O: isopropanol mixture. Samples containing Zn, Cu-Cmt complexes with divalent metal ions were analysed under the following conditions: 20 μL of protein solution injected through a PEEK (polyether heteroketone) column at 40 μl/min; capillary counter-electrode voltage 5 kV for Zn and 3.5 kV for Cu; desolvation temperature 80–110°C; dry gas 6 L/min; spectra collection range 80 0–2500 m/z. The carrier buffer was a 5:95 mixture of acetonitrile:ammonium acetate (15 mM, pH 7.0) for Zn and a 10:90 mixture for Cu. Analysis of apo-Cmt and Cu-Cmt at low pH was performed using a 5:95 mixture of acetonitrile:formic acid at pH 2.4. Under all the conditions assayed, the error associated with the mass measurements was always lower than 0.1%.

Antibodies

ATP7A antibody was a gift from Dr. Michael Petris, University of Missouri. Cox IV and luciferase antibodies were purchased from Abcam. GAPDH antibody was purchased from Santa Cruz. FLAG antibody was purchased from Sigma. Immunofluorescence microscopy and immunohistochemistry were performed as previously described (Ding et al., 2011). All microscopy images were taken using a Zeiss Axio Imager wide field fluorescence microscope (ZEISS).

Bronchoalveolar lavage (BAL) isolation from animals

BAL isolation was performed as described previously (Okagaki et al., 2010), except the fluid was centrifuged and resuspended in ACK lysis buffer (NH_4Cl, $KHCO_3$ and EDTA) to lyse red blood cells, and then washed 3 times with PBS.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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- *C. neoformans* senses a high Cu environment during pulmonary colonization
- *Cryptococcus* Cu detoxification machinery is important for fungal virulence
- *Cryptococcus* metallothioneins are Cu specific detoxification proteins

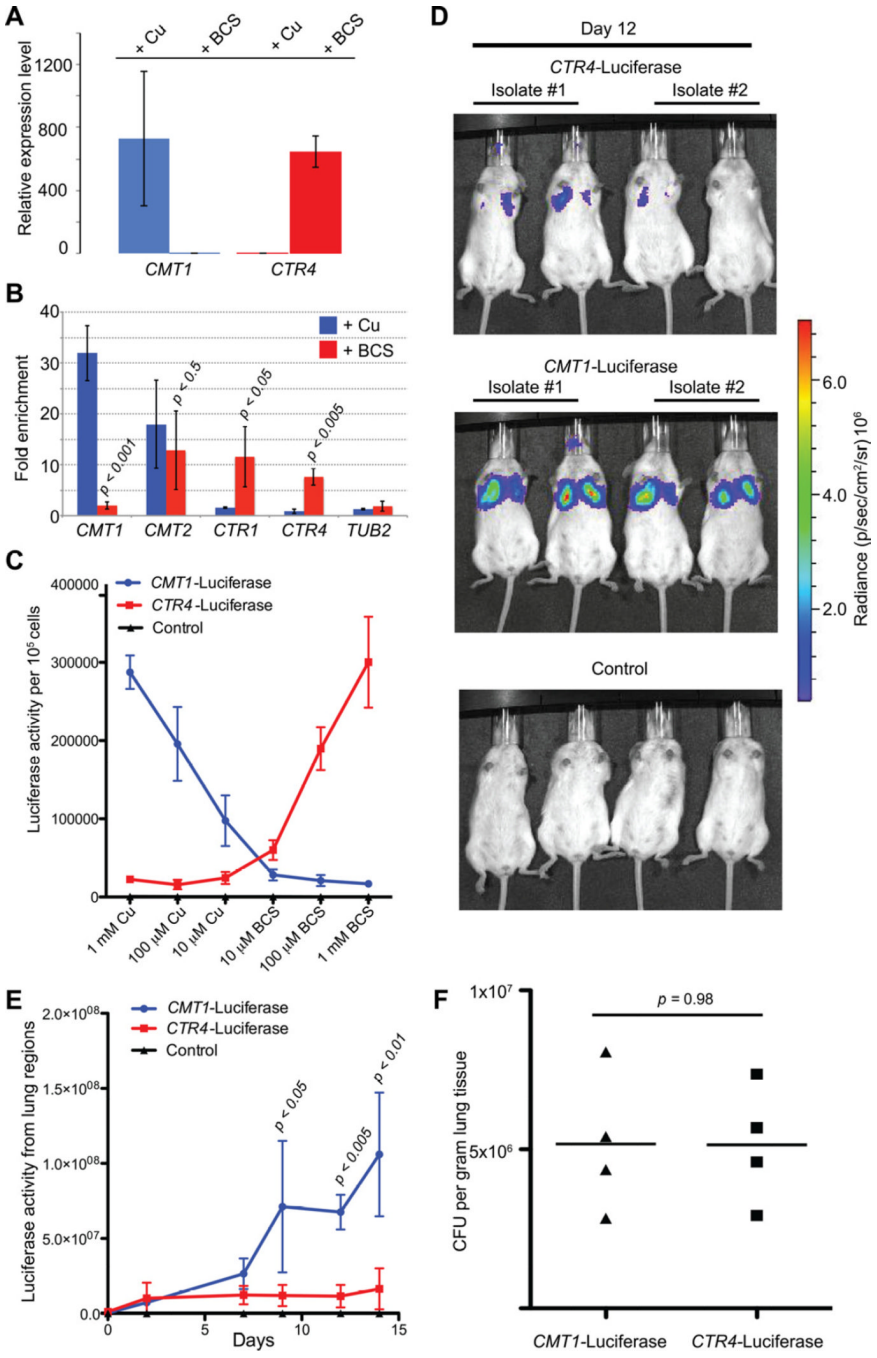

Figure 1. Cu sensing reporter systems in *C. neoformans* (See also Figure S1 and Table S1)

(**A**) Expression of *CMT1* and *CTR4* was quantitated using qRT-PCR. Cell cultures were subcultured in SC medium supplemented with 1 mM Cu or BCS, and incubated at 37°C for 3 hrs. Expression levels were normalized to *ACT1*. Error bars indicate standard deviation (SD).

(**B**) ChIP was performed in *cuf1Δ/CUF1-2xFLAG* strains after growth in the presence of 1 mM Cu or BCS. Quantitative PCR was performed to measure enrichment of promoter sequences from *CMT1*, *CMT2*, *CTR1*, *CTR4* and *TUB2* (negative control). Statistical analysis was performed using the *student t test*. Error bars indicate SD.

(**C**) Luciferase activities from fungal cells harboring reporter genes for *CMT1*-Luciferase, *CTR4*-Luciferase or wildtype (negative control) were quantified. Cells were grown in SC medium supplemented with Cu or BCS, 37°C for 9 hrs. Luciferase activities were measured using the Luciferase Reporter Assay (Qiagen). Error bars indicate SD.
(**D**) Luciferase activities from four mice each infected with *CMT1*-Luciferase, *CTR4*-Luciferase or wild type were measured using live animal imaging. Two independent isolates carrying *CMT1*-Luc or *CTR4*-Luc, or control wild type cells were used for intranasal mouse infections and luciferase activity scans performed at days 0, 2, 7, 9, 12 and 14, and day 12-post infection is shown.
(**E**) Luciferase activity from the lungs of each mouse (in **D**) was measured and analyzed using Living Image 4.2 (Caliper, PerkinElmer). Statistical analysis was performed using the *student t test*. Error bars indicate SD.
(**F**) Fungal cell burden assessed by colony forming units (CFU) from mouse lung homogenates derived from animals in **Figure 1D**.

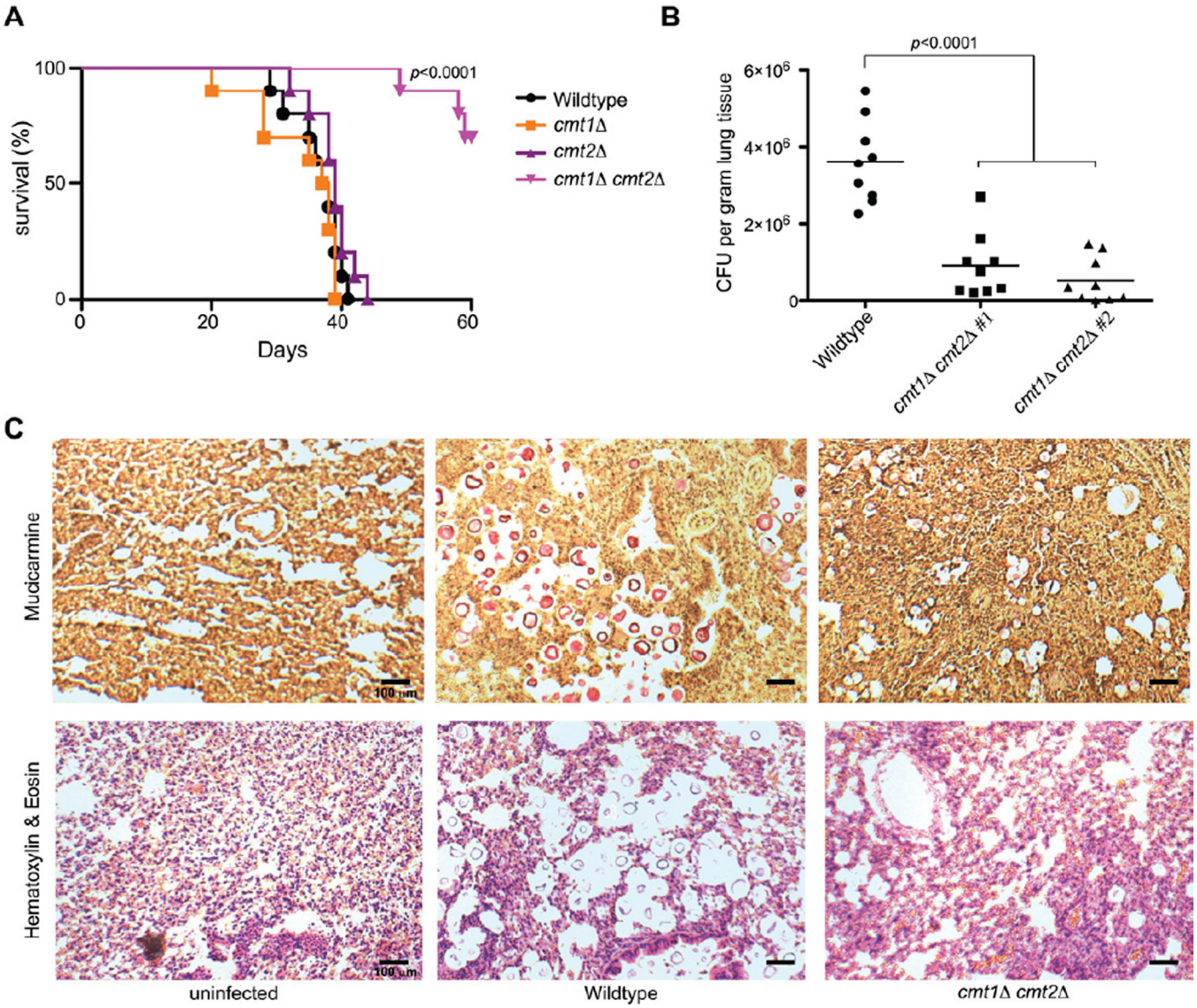

Figure 2. *C. neoformans* metallothioneins are virulence factors (See also Figure S2 and Table S1)

(**A**) Ten A/J female mice were infected intranasally with wildtype, *cmt1Δ, mt2Δ*, or *cmt1Δ cmt2Δ* cells and animals monitored for viability over 60 days. Shown is a Kaplan-Meier survival plot.

(**B**) Mice were infected with wildtype, or two independent *cmt1Δ cmt2Δ* mutants for 14 days and lung tissues were isolated, homogenized and CFU were quantitated and normalized with respect to tissue weight. Statistical analysis was performed using ANOVA.

(**C**) Lung tissue from uninfected, wildtype, or *cmt1Δ cmt2Δ* infected mice were isolated, fixed, and stained with mucicarmine or hematoxylin and eosin.

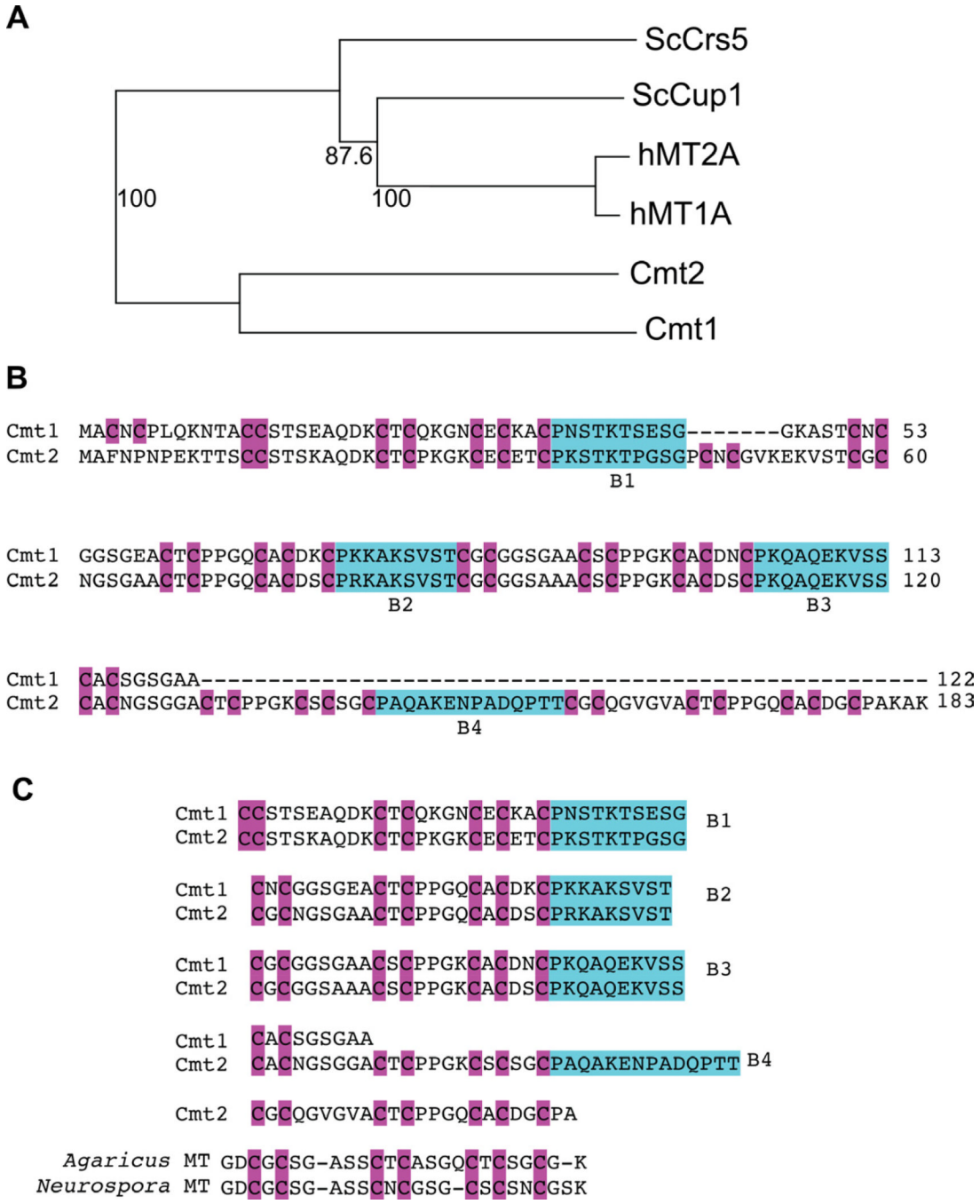

Figure 3. Atypical *C. neoformans* metallothioneins (See also Figure S3)

(**A**). A phylogenetic MT tree was generated as described previously (Ding et al., 2011), with percentage of confidence (bootstrap) shown in numbers. Both *C. neoformans* MTs are distantly related to those from *S. cerevisiae* (Sc) and human (h).

(**B**). Protein sequences from *CMT1* and *CMT2* were aligned. The homologous cysteine residues are shaded in purple, and spacer boxes are shaded in green. Both Cmts contain spacer regions (B1 to B3 for Cmt1 and B1 to B4 for Cmt2). The spacer sequences share high similarity between Cmt1 and Cmt2 and divide each Cmt into multiple cysteine-rich

segments, resulting in a peculiar architecture of 3 Cys-rich segments for Cmt1 and 5 for Cmt2.

(**C**). Cmt1 and Cmt2 share a common motif Cys-X-Cys-X_6-Cys-X-Cys-X_4-Cys-X-Cys-X_2-Cys motif in their Cys-rich segments. These motifs are separated by 3 spacer regions in Cmt1 and 4 in Cmt2, and this motif is similar to that found in MTs in other fungi such as *Agaricus* and *Neurospora*.

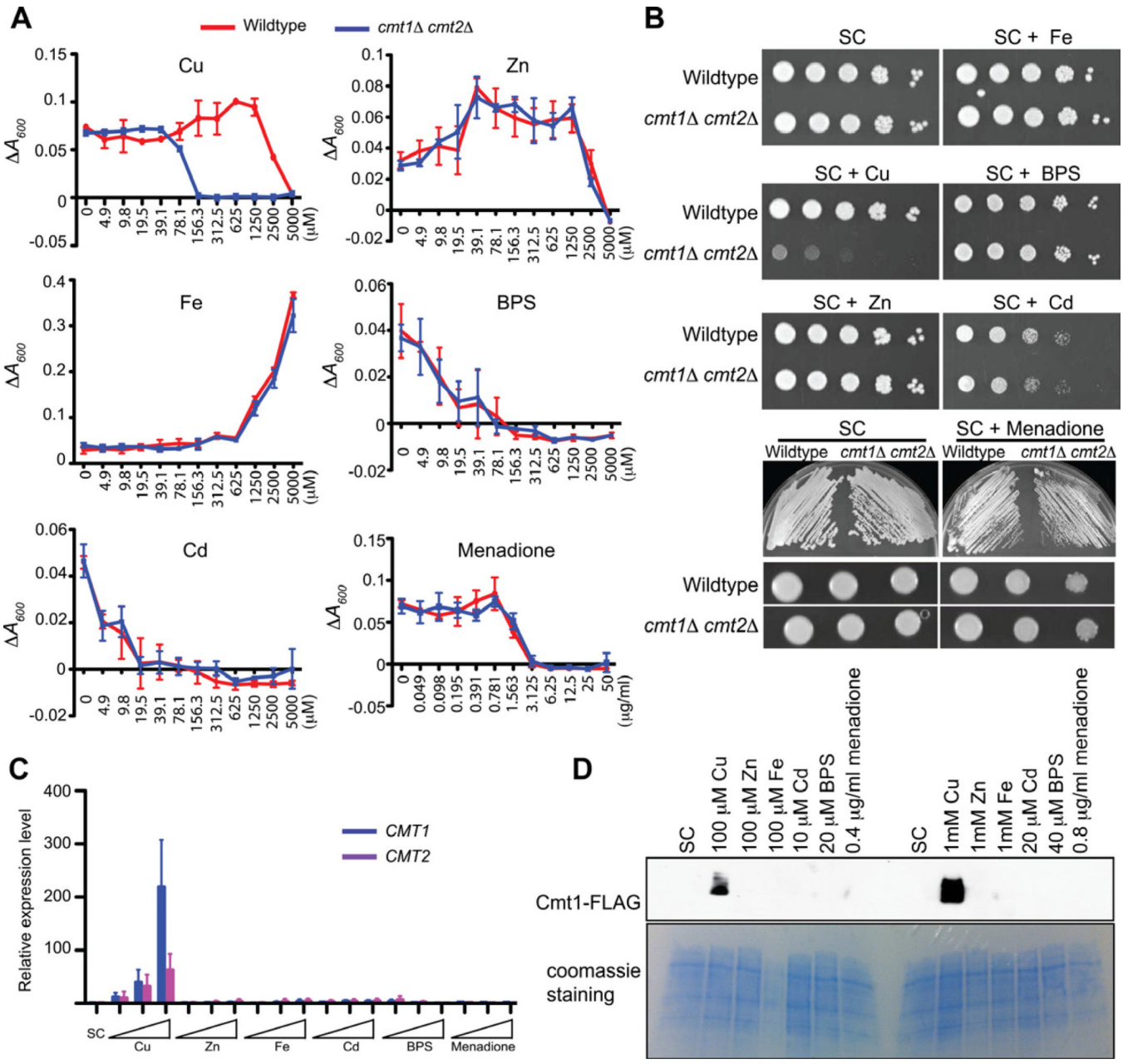

Figure 4. *C. neoformans* MTs are Cu responsive Cu detoxification proteins (See also Table S1)

(A). *C. neoformans* cell growth assays were performed in SC medium in 96 well plates. Cell cultures of wildtype and *cmt1*Δ *cmt2*Δ were diluted to an A_{600} of 0.002, supplemented with metals. A_{600} was measured after overnight growth. ΔA_{600} was calculated by subtracting from blank (medium without cells). Graphs show average of three biological replicates. Error bars indicate SD.

(B). *C. neoformans* metal sensitivity assays on agar medium. Cell cultures of wild type and *cmt1*Δ *cmt2*Δ cells were diluted in water to an A_{600} of 1.0. 10-fold serial dilutions cells were spotted onto SC agar or agar supplemented with 400 μM Cu, Zn, Fe, 100 μM Cd, 40 μM BPS, or 10 μg/ml menadione. Plates were incubated for 2 days (6 days for menadione spotting assay) and photographed.

(C). Expression of *CMT1* and *CMT2* was quantitated by qRT-PCR. Cell cultures were diluted to an A_{600} of 0.2 in SC medium at 37°C for 1hr, supplemented with the indicated concentrations (selected according to the results from Figure 4A. 10, 100, 1000 μM For Cu, Zn, Fe; 5, 10, 20 μM for Cd; 10, 20, 40 μM for BPS; 0.2, 0.4, 0.8 μg/ml for menadione). Error bars indicate SD.

(D). Protein expression of Cmt1-FLAG was confirmed by immunoblotting. Cells were grown as described as Figure 4C. Protein extracts were treated with TCEP, resolved by SDS-PAGE and anti-FLAG mouse antibody was used for immunoblotting. Coomassie staining was used as a loading control.

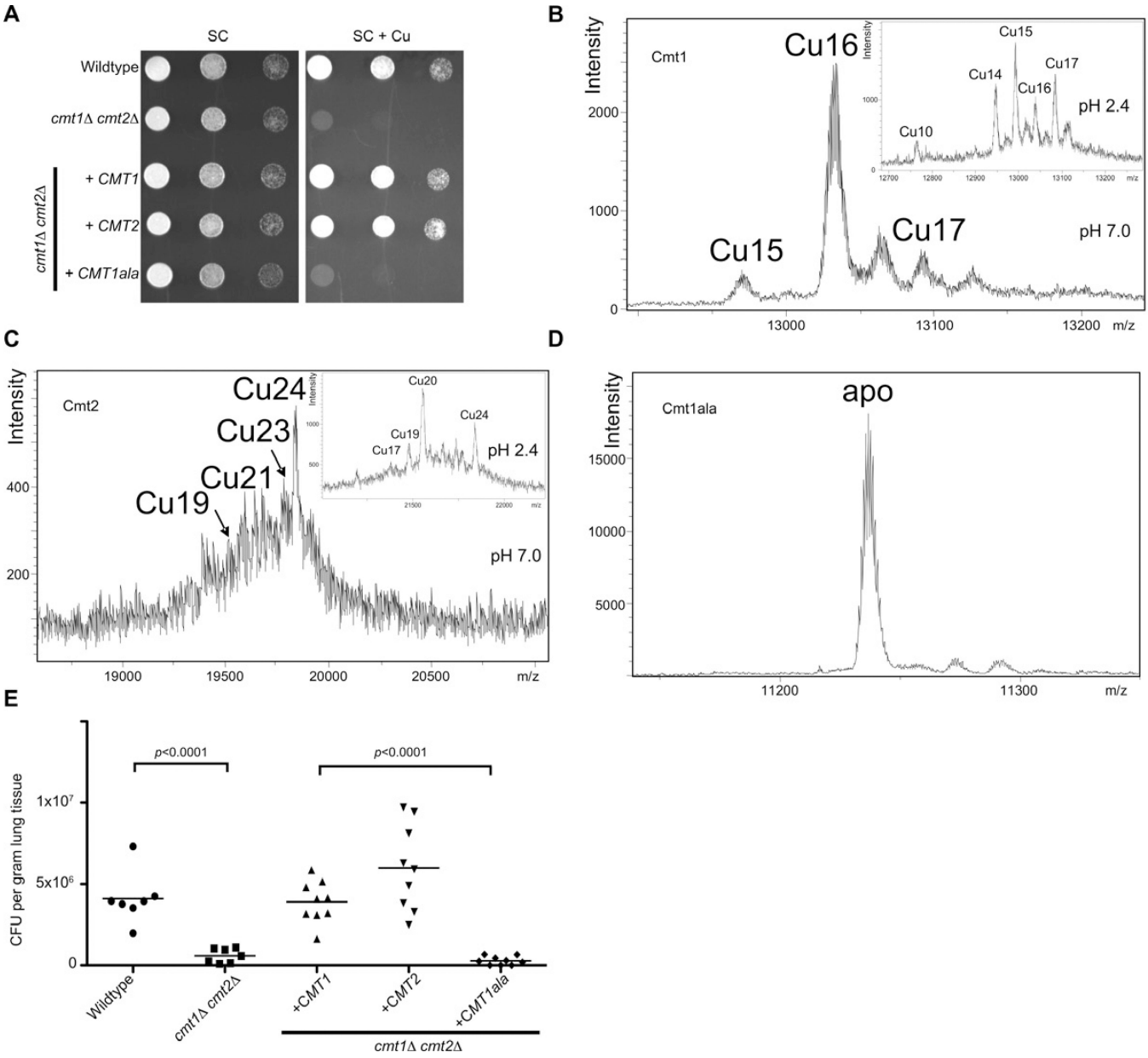

Figure 5. *C. neoformans* MT Cu binding capacity is critical for virulence (See also Figure S4 and Table S1)

(**A**) Cu-resistance growth assays in SC medium supplemented with 1 mM Cu with the *cmt1Δ cmt2Δ* mutant expressing *CMT1*, *CMT2* or the *CMT1*ala mutant. Growth assays were performed as described in Figure 4B.

(**B**) ESI-MS spectra at pH 7.0 and pH 2.4 (insets) of purified Cmt1, (**C**) Cmt2 and the Cmt1ala mutant (**D**).

(**E**) Lung tissue fungal burden (colony forming units, CFU) from *cmt1*Δ *cmt2*Δ cells transformed with plasmids expressing *CMT1*, *CMT2* or *CMT1ala*. Experiments and statistical analysis were performed as described in Figure 2B.

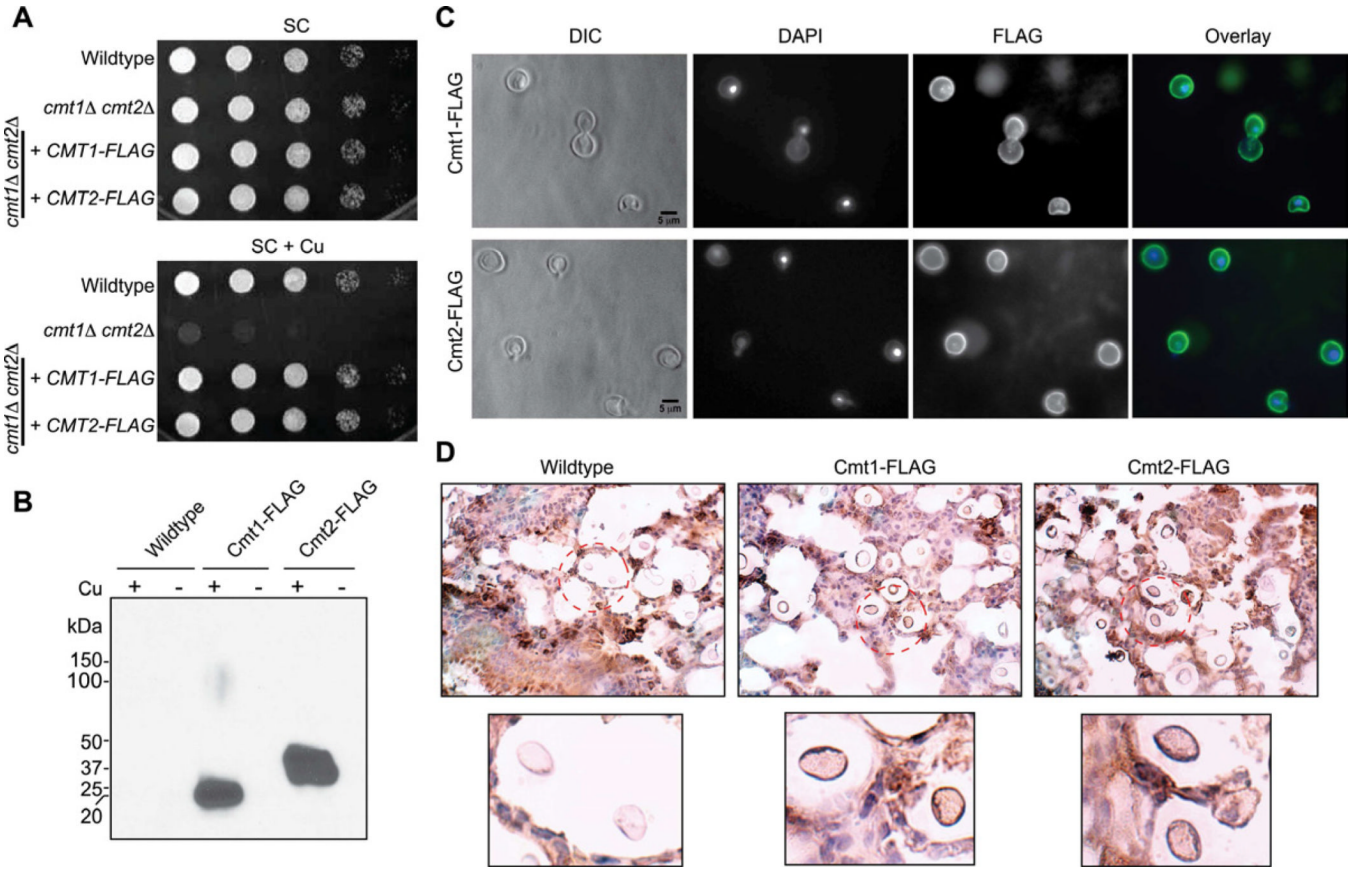

Figure 6. *C. neoformans* MTs concentrate at the cell periphery (See also Table S1)

(A) *cmt1Δ cmt2Δ CMT1-FLAG* and *cmt1Δ cmt2Δ CMT2-FLAG* cells were generated and Cu sensitive phenotype assays performed by spotting 10-fold serial dilutions on SC agar or SC agar supplemented with 1 mM Cu.

(**B**) Immunoblotting confirmed expression of Cmt1-FLAG and Cmt2-FLAG. *C. neoformans* cells (wildtype, *cmt1Δ cmt2Δ CMT1-FLAG* and *cmt1Δ cmt2Δ CMT2-FLAG*) were incubated in the presence of 200 μM Cu (+) or BCS (−) in SC medium for 3 hrs and immunoblotting was performed as described in Figure 4D. Ponceau S staining confirmed equal protein loading.

(**C**) Cmt1-Flag and Cmt2-Flag proteins localized by indirect immunefluorescence microscopy with anti-Flag antibody and the DNA stains DAPI for localizing nuclei.

(**D**) Lung tissue from mice infected (14 day-post infection) with wildtype, *cmt1Δ cmt2Δ CMT1-FLAG* or *cmt1Δ cmt2Δ CMT2-FLAG* was analyzed by H&E staining and immunohistochemistry using anti-Flag antibody.

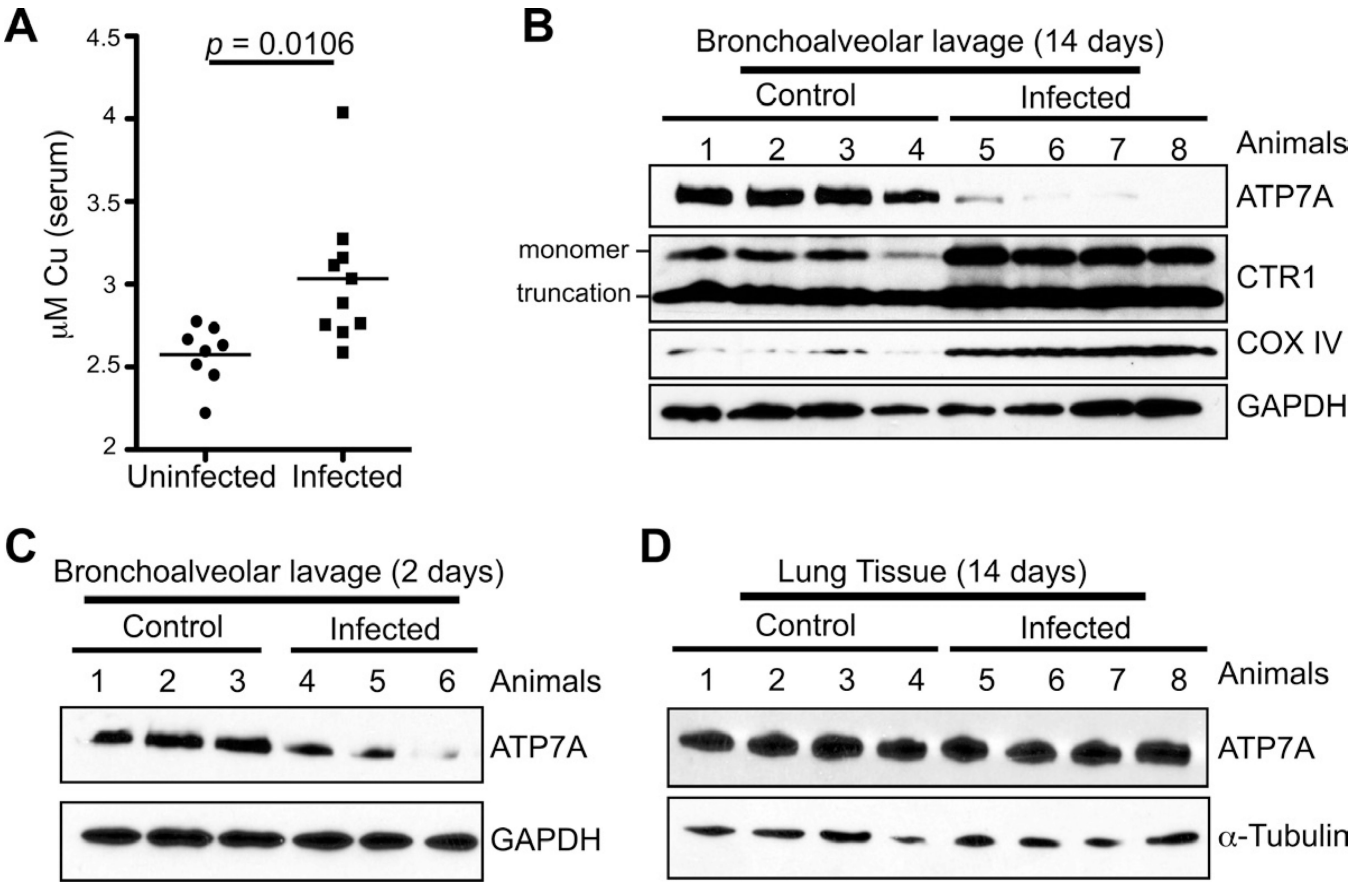

Figure 7. *C. neoformans* infection alters host Cu transport machinery

(**A**) Mice were infected with wild type *C. neoformans* cells and serum was isolated at day 14-post infection. Cu were measured using ICPMS and shown for uninfected and *C. neoformans* infected mice.

(**B**) Bronchoalveolar lavage cells (BAL) were isolated from uninfected mice and mice infected with wildtype cells 14 post-infection. Protein was extracted from the BAL and ATP7A, Ctr1, Cox IV and GAPDH levels analyzed by SDS-PAGE and immunoblotting.

(**C**) BAL protein extract was analyzed 2 days after infection by immunoblotting for ATP7A and GAPDH.

(**D**) Lung tissue from mice 14-days after infection was analyzed for ATP7A and tubulin levels by immunoblotting.