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The Cu regulon of the human fungal pathogen *Cryptococcus neoformans* H99: Cuf1 activates distinct genes in response to both Cu excess and deficiency

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Summary

Cryptococcus neoformans is a human fungal pathogen that is the causative agent of cryptococcosis and fatal meningitis in immuno-compromised hosts. Recent studies suggest that copper (Cu) acquisition plays an important role in *C. neoformans* virulence, as mutants that lack Cuf1, which activates the Ctr4 high affinity Cu importer, are hypo-virulent in mouse models. To understand the constellation of Cu-responsive genes in *C. neoformans* and how their expression might contribute to virulence, we determined the transcript profile of *C. neoformans* in response to elevated Cu or Cu deficiency. We identified two metallothionein genes (*CMT1* and *CMT2*), encoding cysteine-rich Cu binding and detoxifying proteins, whose expression is dramatically elevated in response to excess Cu. We identified a new *C. neoformans* Cu transporter, CnCtr1, that is induced by Cu deficiency and is distinct from CnCtr4 and which shows significant phylogenetic relationship to Ctr1 from other fungi. Surprisingly, in contrast to other fungal, we found that induction of Cn*CTR1* and Cn*CTR4* expression under Cu limitation, and *CMT1* and *CMT2* in response to Cu excess, are dependent on the CnCuf1 Cu metalloregulatory transcription factor. These studies set the stage for the evaluation of the specific Cuf1 target genes required for virulence in *C. neoformans*.

Introduction

Cryptococcus species are air-borne human fungal pathogens that are the causative agent of cryptococcosis, a life-threatening infection that occurs particularly in patients with impaired immunity due to cancer chemotherapy, HIV-AIDS, diabetes or immunosuppression from organ transplants, as well as in immuno-competent individuals (Aberg *et al.*, 1999, Casadevall A, 1998, Dromer *et al.*, 1988, Henderson *et al.*, 1982). For the past several years *Cryptococcus* species have emerged as significant fungal pathogens and recent outbreaks of *Cryptococcus* have occurred in the Pacific Northwest of North America and in Europe, Australia and elsewhere (Byrnes *et al.*, 2009, Byrnes *et al.*, 2010, Carriconde *et al.*, 2011, Fraser *et al.*, 2005, Hoang *et al.*, 2004).

A number of *C. neoformans* virulence factors have been previously reported, including the generation of its polysaccharide-rich capsule, melanization, iron (Fe) acquisition and

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phenotypic switching (Liu *et al.*, 1999, Salas *et al.*, 1996, Chang & Kwon-Chung, 1994, Jung *et al.*, 2006, Jung *et al.*, 2008). Recent reports suggest that the trace element copper (Cu) may play a critical function in the virulence of *C. neoformans* as assayed in mouse models of intravenous infection (Waterman *et al.*, 2007). First, the ability to produce the pigment melanin is dependent on a key Cudependent enzyme laccase, which is predicted to be synthesized and loaded with Cu in the secretory compartment (Williamson, 1994, Walton *et al.*, 2005, Zhu & Williamson, 2004, Kim *et al.*, 2008). The importance of melanin in *C. neoformans* virulence is thought to be due to its role in oxygen radical detoxification in macrophages, its function in cell wall integrity and protection from high temperatures and other stresses (Liu et al., 1999, Zhu *et al.*, 2001, Zhu & Williamson, 2004, Garcia-Rivera *et al.*, 2005). Importantly, *C. neoformans* carrying a mutation in the laccase-encoding gene *LAC1* is significantly less virulent than isogenic wild type strains (Noverr *et al.*, 2004, Zhu et al., 2001, Zhu & Williamson, 2004). Moreover, *C. neoformans* strains with mutations in other genes encoding Cu acquisition and distribution proteins that include the *CLC* chloride channel and the *CCC2* secretory compartment Cu pump show reduced virulence and Cu deficiency phenotypes (Walton et al., 2005, Zhu & Williamson, 2003, Zaballa *et al.*, 2010, Stoj *et al.*, 2007). As Cu is also a critical co-factor for the multi-Cu oxidases involved in high affinity Fe^{2+} uptake, and Fe acquisition is a virulence factor, this may be a second the role of Cu in virulence (Stoj et al., 2007, Zaballa et al., 2010, Jung & Kronstad, 2011, Jung *et al.*, 2009). Moreover, deletion of the gene encoding Cuf1, previously suggested to be a Cu-deficiency sensing transcription factor that activates expression of the *C. neoformans* Ctr4 Cu importer, rendered cells both Cu deficient and with significantly reduced proliferation in the brain and spleen in a mouse model of *C. neoformans* intravenous infection (Waterman et al., 2007, Stoj et al., 2007, Zaballa et al., 2010, Lin *et al.*, 2006).

While Cu homeostasis genes, a Cu responsive transcription factor and Cudependent proteins have been implicated in *C. neoformans* virulence, the genome wide responses to Cu deficiency and Cu excess have not been described. Here we report the characterization of the transcriptome of *C. neoformans* in response to both Cu deficiency and Cu excess. We identified two genes encoding members of the Cu detoxifying metallothionein proteins, a gene encoding a new high affinity Cu import protein as well as additional Cu responsive transcripts. Surprisingly, in contrast to other characterized fungi in which dedicated Cu sensing transcription factors activate gene transcription under either Cu deficiency or excess, the Cuf1 Cu-sensing transcription factor of *C. neoformans* is required for both regulatory responses. This genome-wide characterization of the *C. neoformans* Cu deficiency and Cu excess regulons sets the stage for a systematic analysis of the role of Cu in the virulence of this important human fungal pathogen.

Results

Identification of molecular markers for Cu regulation in *C. neoformans*

To establish the conditions for transcriptome analysis of *C. neoformans* in response to Cu deficiency or Cu excess, we sought to identify Cu responsive genes that would serve as molecular markers elevated in response to exogenous Cu levels. Computational interrogation of the *C. neoformans* genome sequence identified two genes potentially encoding the Cu-binding and detoxifying metallothionein proteins, which we designate as *Cryptococcus* metallothioneins (*CMTs*): *CMT1* (*CNAG_05549*) and *CMT2* (*CNAG_00306*). In other fungal species, expression of genes encoding metallothionein proteins is robustly induced by high levels of extracellular Cu and in mammals MT genes are transcriptionally induced by a broader spectrum of metals (Butt *et al.*, 1986, Ecker *et al.*, 1986, Jeyaprakash *et al.*, 1991, Kagi & Hunziker, 1989, Thiele, 1988, Thiele *et al.*, 1986, Balamurugan & Schaffner, 2006). We examined the expression level of *CMT1* and *CMT2* mRNAs in response to elevated Cu by RNA blotting experiments with the corresponding

CMT1 and *CMT2* complementary DNAs. The expression of *CMT1* mRNA was dramatically induced in a dose-dependent manner in response to high concentrations of Cu, and basal *CMT1* transcript levels were repressed in the presence of the Cu(I) chelator BCS (low Cu conditions) (Figure 1A). To investigate the kinetics of induction of *CMT1* and *CMT2* mRNA levels, we incubated *C. neoformans* cells with 1 mM Cu and ascertained mRNA levels in a time course experiment. Expression of *CMT1* and *CMT2* in response to exogenous Cu is dramatically and rapidly induced within the first 30 min of incubation at this concentration of Cu and sustained over at least 5 hours in the chronic presence of Cu (Figure 1B).

To characterize the conditions for *C. neoformans* transcriptome analysis in response to Cu deficiency, the expression of Cn*CTR4*, encoding a previously identified protein with homology to the high affinity Cu(I) importer family, was ascertained in the presence of Cu or BCS. In contrast to the expression of the two *C. neoformans* metallothinein genes, Cn*CTR4* mRNA levels are repressed under high Cu conditions and highly induced in the presence of 1 mM BCS (Figure 1A). The time course analysis indicates that elevation of Cn*CTR4* mRNA steady state levels in response to Cu deficiency was slower than that of *CMT1* and *CMT2* in response to elevated Cu and was maximal after approximately 2 to 3 hr in the presence of BCS (Figure 1C).

Transcript profiling of *C. neoformans* in response to Cu availability

We performed transcriptome profiling to facilitate an understanding of global changes in gene expression in response to Cu availability in *C. neoformans*. Cells were incubated in the presence of 1 mM Cu or 1 mM BCS for 3 hr, as at these conditions cells demonstrated robust gene expression of *CMT1* and Cn*CTR4* (Figure 1). RNA samples were amplified, labeled with Cy3 or Cy5 and hybridized onto *C. neoformans* serotype A and D DNA microarray slides. The microarray probes were remapped based on the *C. neoformans* genome.

A total of twenty-three mRNA species showed significant changes in abundance after 3 hr under the specified culture conditions, including those corresponding to 14 genes induced by 1 mM Cu and 9 genes induced in the presence 1 mM BCS (Table 1). As expected, and serving as positive controls for this experiment, expression of the *CMT1* and *CMT2* genes is strongly elevated under high Cu conditions, whereas elevation of Cn*CTR4* expression is evident as a positive control for Cu deficiency conditions, in agreement with the RNA blotting results (Figure 1A). Among the high Cu induced genes, we also observed that genes predicted to encode isochorismatase (*CNAG_02427*), phosphatidylserine decarboxylase (*CNAG_00834*) and oxidoreductase (*CNAG_01102*) are significantly elevated in their expression. Under Cu limitation conditions we observed elevated expression of *CNAG_00110*, potentially encoding a Rho GTPase, and *CNAG_00876* (encoding a potential ferric, cupric-chelate reductase) as well as other genes encoding proteins with potentially known and unknown functions (Table 1).

Identification of a new high affinity Cu importer in *C. neoformans*

Interestingly, we detected a mRNA encoding a new potential Cu transporter (*CNAG_07701*) whose expression is induced under conditions of Cu limitation (Table 1). Using RNA blotting we observed that expression of *CNAG_07701* is lower in the presence of exogenous Cu compared with Cu deficient conditions, and the basal levels of mRNA corresponding to this putative Cu transporter are significantly higher than that of *CTR4* in *C. neoformans*, as the mRNA can be detected even in the presence of 1 mM Cu (Figure 1A). By analogy, in *S. cerevisiae* the expression of two plasma membrane localized Cu transporters, *CTR1* and *CTR3*, is positively and coordinately regulated by low Cu growth conditions via the Mac1

transcription factor (Jungmann *et al.*, 1993, Labbe *et al.*, 1997, Zhu *et al.*, 1998, Jensen *et al.*, 1998). Moreover, the *S. cerevisiae* vacuolar Cu transporter, *CTR2* is not regulated by Cu deficiency, but rather is induced by low Fe (Rees *et al.*, 2004). We demonstrated here the presence of a putative *CTR2* orthologue (*CNAG_01872*) in *C. neoformans* (Figure 3), the expression of which is not regulated by exogenous Cu levels (Figure 1A).

Unlike *S. cerevisiae*, the majority of genes in the *C. neoformans* genome contain introns (Loftus *et al.*, 2005). This may cause ambiguous predictions of coding DNA sequences when using strictly computational approaches. To unambiguously assign polypeptide sequences to *CNAG_07701* and Cn*CTR4*, we sequenced the cDNA of these transcripts using the RLM-RACE method (Supplemental file 2). The cDNA sequences demonstrated that the encoded protein sequences possess methionine rich regions at the amino-terminus, and in one of the transmembrane regions, including M-X_2-M and M-X-M motifs at the amino-terminus, and an M-X_3-M motif (essential for function in the Ctr1 family of Cu transporters) within a predicted transmembrane domain (Figure 2A). Phylogenetic analysis infers that *CNAG_07701* is a homologue of *CTR1* from *S. cerevisiae* (Figure 3), therefore we named the polypeptide encoded by this Cu-deficiency induced transcript Cn*CTR1*.

To ascertain information about the function, expression and localization of Cn*CTR1*, the genomic DNA sequence encoding this protein was modified to introduce sequences encoding a FLAG epitope tag at the amino- or carboxyl-termini, respectively, under the control of the endogenous promoter. The plasmids containing DNA sequences encoding FLAG-tagged CnCtr1 were transformed into a Cn*ctr1* strain and the CnCtr1 protein detected by immunoblotting. We detected a polypeptide between 25 to 37 KDa for both the amino- and carboxyl-terminal-tagged CnCtr1, with the molecular weight of the detected protein similar to the computationally predicated mass of 32.7 KDa (Figure 2B). These results demonstrated that robust signals were detected from cells grown in the presence of 1 mM BCS compared with cells treated with Cu. The immunoblot results strongly correlate with the mRNA expression pattern of Cn*CTR1*, demonstrating that expression of both Cn*CTR1* mRNA and protein is regulated by Cu availability and induced during Cu deficiency (Figures 1A and 2B).

To further investigate the function of the *C. neoformans* Ctr1 and Ctr4 Cu transporters, we carried out complementation experiments in a *ctr1Δ/ctr3Δ* strain from *S. cerevisiae* with the Cn*CTR1* or Cn*CTR4* cDNA sequences. The cDNA sequences from Cn*CTR1* and Cn*CTR4* were amplified and subcloned in plasmid p426GPD, with expression driven by the Sc*GPD* promoter, and transformed into the Sc*ctr1Δ/ctr3Δ* strain. Expression of the cDNA sequence from Cn*CTR1* or Cn*CTR4* rescues the cell growth defect of a Sc*ctr1Δ/ctr3Δ* strain when using glycerol and ethanol as sole carbon sources, as their use in mitochondrial oxidative phosphorylation is Cu-dependent (Figure 2C). These observations are consistent with the Cn*CTR1* and Cn*CTR4* proteins functioning as independent Cu importers in the *S. cerevisiae* background.

We determined the localization of the Cn*CTR1* protein by independently expressing a mCherry or FLAG tagged Ctr1 allele. We found that the function of Cn*CTR1* is abolished when tagged with mCherry at the amino-terminus, and the carboxyl-terminal mCherry tag affects CnCtr1 protein trafficking, which showed localization on plasma membrane, endoplasmic reticulum membrane, and in the vacuolar lumen (data not shown). Using Ctr1-Flag strain, we performed immunofluorescence microscopy and detected a fluorescent signal on the plasma membrane and inside the vacuolar lumen (Figure 2D), implying that Cn*CTR1* localizes on the plasma membrane, and may be degraded in the vacuole.

Phylogenetic analysis of the *C. neoformans* Cu transporters

A previous analysis suggested that *CNAG_07701* encodes a Cu transporter which was designated *CTR2* (Chun & Madhani, 2010). This inference is primarily based on homology to other eukaryotic Cu transporters using a computationally predicted protein sequence of *CNAG_07701* from the genomic DNA. To infer the evolutionary history of *CNAG_07701* we reconstructed a fungal Ctr phylogeny analysis. Proteomes were obtained for more than 100 fungal genomes (as described in Material and Methods) and these formed our database. Amino acid sequences were obtained for *Saccharomyces cerevisiae* Ctr1, Ctr2 and Ctr3 and these three proteins, along with the translated sequence from the cDNA sequence of *CNAG_07701* were used as query sequences in a bidirectional database search (see methods). We initially considered bidirectional database hits as orthologs while one-way hits were labeled as homologs. Using our database search criteria we could not locate a homolog of *CNAG_07701* in the genome of *S. cerevisiae.* Furthermore with the exception of *Yarrowia lipolytica*, we failed to locate orthologs of *CNAG_07701* in any of the *Saccharomycotina* species represented in this analysis (supplemental file 3, Figure S1). However closer inspection of our database search results indicated that Ctr1 and CNAG_07701 may be distantly diverged homologs. We based this initial observation on the fact that Ctr1 and CNAG_07701 have the same top hit in twenty of the species used in this analysis (not shown).

All 332 Ctr homologs were extracted from our database, aligned and a global Ctr phylogeny was reconstructed (supplemental file 3, Figure S1). For display purposes a representative Ctr phylogeny was also inferred (Figure 3). Based on our phylogenies three clades are evident. Ctr2 and Ctr3 homologs form monophyletic clades (Figure 3 and supplemental file 3, Figure S1) and are more closely related to one another than they are to the Ctr1 and CNAG_07701 homologs (Figure 3, 90% bootstrap support). For clarity, the presence and absence of CTR orthologs are displayed on a fungal species tree (supplemental file 3, Figure S2)

The phylogenetic position of the *Saccharomycotina* Ctr1 homologs is interesting. They are grouped beside the CNAG_07701 homologs (Figure 3 and supplemental file 3, Figure S1), which is not surprising as our bidirectional database search showed low levels of similarity between these proteins. Therefore based on our database search and subsequent phylogenetic analysis, we consider Ctr1 and CNAG_07701 as divergent homologs and will refer to both groups as Ctr1 herein. Interestingly the Saccharomycotina Ctr1 homologs are not grouped beside the remaining *Ascomycota* Ctr1 homologs (Figure 3 and supplemental file 3, Figure S1). A number of possible scenarios can explain this inference. For example, the last common ancestor of all fungal species represented in this analysis may have had two copies of Ctr1. These may have diverged through neofunctionalization or subfunctionalization followed by a gene loss in the common ancestor of the S*accharomyctina* species, while differential independent losses could have also occurred in the remaining ascomycetes, basidiomycetes and chytridiomycetes ancestors. However, the most parsimonious inference is one where there is a duplication of Ctr1 at the base of the *Saccharoycotina* clade followed by divergence and loss of the original Ctr1 ortholog. This loss would have occurred after the speciation from *Y. lipolytica* as it possesses orthologs of both Ctr1 and CNAG_07701 (Figure 3 and supplemental file 3, Figure S1).

C. neoformans Cu transporters function in Cu homeostasis and melanin production

To ascertain the contributions of select Cu-responsive genes to Cu homeostasis, gene disruptions and phenotypic analyses were carried out. The open reading frame (ORF) of Cn*CTR1* or Cn*CTR4* was replaced by inserting a NAT^R or Neo^R knock-out cassette, respectively. In *S. cerevisiae*, disruption of both *CTR1* and *CTR3* causes growth defects in Cu deficiency conditions (Pena *et al.*, 2000, Knight *et al.*, 1996). A Cn*ctr1Δ* strain showed a

severe growth defect compared with the wild type parental strain and the Cn*ctr1Δ* strain that was reconstituted with a FLAG-epitope tagged CnCtr1 allele, when cultured on medium supplemented with 1 mM BCS (Figure 4A). In contrast, deletion of the Cn*CTR4* coding region alone had no effect on cell growth under these Cu deficiency conditions, comparing with the wildtype parental strain (Figure 4A). Interestingly, the growth of a Cn*ctr1Δ*/*ctr4Δ* strain, in which both putative high affinity Cu transporters were deleted, was severely inhibited by low Cu conditions, (Figure 4A). These data suggest that CnCtr1 and CnCtr4 have distinct contributions to Cu import, which may be related to their distinct basal mRNA levels of expression (Figure 1A).

Melanin production has been reported to be an important factor for *C. neoformans* virulence in both macrophage survival assays *in vitro* and in mouse infection models (Liu et al., 1999, Noverr et al., 2004). As laccase, encoded by the Cn*LAC1* gene, is a Cu binding protein that is critical for melanin synthesis, which is a Cu dependent process (Williamson, 1994). We have presented evidence that CnCtr1 and CnCtr4, structurally similar to other fungal and mammalian high affinity Cu importers, have functional properties of Cu transporters in *C. neoformans*. Hence deleting both the CnCtr1 and CnCtr4 Cu transporters would be predicted to result in a defect in melanin formation. We show here that single deletions of either Cn*CTR1* or Cn*CTR4* has no obvious melanin production defect when cells are propagated on DOPA agar or DOPA agar supplemented with a low concentration of BCS (Figure 4B). Disrupting both Cn*CTR1* and Cn*CTR4* simultaneously results in a modest reduction in melanin formation on DOPA agar and severely reduces melanin formation under low Cu conditions (10 μM BCS) (Figure 4B).

Cu is required for Fe uptake, and the activity of a high affinity Fe transporter, Fet3, from *S. cerevisiae* depends on Cu (Stoj et al., 2007, Zaballa et al., 2010, Kaplan, 2002). We therefore tested Cu transporter mutants from *C. neoformans* in Fe iron conditions. The absence of Cn*CTR1* or Cn*CTR4* has no significant defect on cell growth in the presence of BPS. Whereas, Cn*ctr1Δ/ctr4Δ* and Cn*cuf1Δ* strains, which have Cu uptake deficiency, demonstrate growth defects under Fe iron conditions (Supplemental file 3, Figure S3).

Identification of *C. neoformans* metallothioneins

The cDNA sequences from *CMT1* and *CMT2* were also sequenced using the RLM-RACE method (Supplemental file 2) and comparison with the genomic sequence showed that the *CMT1* gene contains 5 introns and *CMT2* gene contains 7 introns. The encoded protein sequences from the *CMT1* and *CMT2* cDNAs contain multiple cysteine-rich regions, comprising approximately 20% of total protein residues for each metallothionein, as is typical for most fungal and mammalian metallothioneins (Winge *et al.*, 1985, Butt *et al.*, 1984b, Kagi & Hunziker, 1989, Szczypka & Thiele, 1989) (Figure 5A). However, the Cmt1 and Cmt2 metallothioneins are surprisingly longer than those from *S. cerevisiae* and *C. albicans*, where Cmt1 and Cmt2 proteins contain 122 aa and 183 aa, respectively, in comparison to 63 aa in *S. cerevisiae* Cup1 and 34 aa in *C. albicans* Cup1 (Butt *et al.*, 1984a, Oh *et al.*, 1999).

To investigate the function of the two putative *C. neoformans* metallothioneins, we carried out complementation experiments in an *S. cerevisiae cup1Δ* strain by expressing the *CMT1* or *CMT2* cDNAs in plasmid p426GPD. Sc*cup1Δ* mutants transformed with the control vector (p426GPD) showed a severe growth defect in the presence of 200 μm $CuSO_4$ (Figure 5B). However, expression of the *CMT1* or *CMT2* cDNAs in this strain rescued the cell growth of Sc*cup1Δ* on medium supplemented with Cu, suggesting that both CMT proteins function similarly to ScCup1 in protecting cells from Cu toxicity.

Previous reports demonstrated that, in a number of fungal species, metallothioneins protect cells from Cu toxicity (Ecker et al., 1986, Oh et al., 1999, Hamer *et al.*, 1985). We generated *cmt1Δ*, *cmt2Δ* and *cmt1Δ/cmt2Δ* deletion strains and compared their ability to grow on medium containing high Cu levels with the isogenic wild type parental strain. Deletion of *CMT1* or *CMT2* individually had no clear effect on cell growth in the presence of elevated levels of Cu (Figure 5C). In contrast, while a *cmt1Δ/cmt2Δ* mutant grew indistinguishably from the parental wild type strain on normal media or that supplemented with BCS, its growth was severely inhibited in the presence of 1 mM Cu (Figure 5C). While Cu(I) binding studies with the purified Cmt1 and Cmt2 proteins have not been conducted, their sequence similarity, ability to complement the Cu sensitivity of a *cup1Δ* strain and the results of endogenous gene deletion experiments indicate that they are likely to be metallothioneins and play a major role in Cu detoxification.

CnCuf1 regulates the expression of both Cu transporters and metallothioneins

The baker's yeast *S. cerevisiae* uses distinct and dedicated Cu metalloregulatory transcription factors to activate the expression of the *CTR1* and *CTR3* high affinity Cu(I) uptake machinery (Mac1) under Cu deficiency, and the *CUP1* and *CRS5* metallothionein genes in response to Cu excess (Ace1) (Jungmann et al., 1993, Labbe et al., 1997, Pena *et al.*, 1998, Thiele, 1988, Culotta *et al.*, 1994).

Interestingly, previous reports suggest that *C. neoformans cuf1Δ* mutants display both Cu deficiency as well as Cu sensitivity phenotypes as compared to the parental wild type strain (Lin et al., 2006, Jiang *et al.*, 2011). To understand the molecular basis for this duality of Cu phenotypes, we generated a Cn*cuf1Δ* strain by replacing the wild type Cuf1 allele with a neomycin resistance marker. Additionally, a wild type copy of the Cuf1 gene was integrated into the genome of a *cuf1Δ* strain, generating a reconstituted strain. We confirmed that a Cn*cuf1Δ* strain is unable to grow under both Cu overload and deficiency conditions, which resembles a composite of the growth phenotypes of Cn*ctr1Δ/ctr4Δ*cells under Cu deficiency conditions and *cmt1Δ/cmt2Δ* cells in high Cu (Figure 4A and 5C). Therefore, we tested whether CnCuf1 might regulate expression of Cn*CTR1*, Cn*CTR4*, *CMT1* and *CMT2* under different Cu conditions. Total RNA was isolated from the wild type parental strain and two independent Cn*cuf1Δ* strains grown in the presence of elevated Cu or BCS and RNA blotting was carried out to assess transcript levels corresponding to these genes. As shown in Figure 6A, the levels of *CMT1* and *CMT2* mRNA are dramatically reduced in Cn*cuf1Δ* strains in the presence of 1 mM Cu, as compared to wild type. It is noteworthy that there is a low level of residual Cu-stimulated elevation of *CMT1* and *CMT2* mRNA steady state levels in the Cn*cuf1Δ* strains, but the precise mechanisms for this are not yet clear. The expression of Cn*CTR1* was also decreased in Cn*cuf1Δ* strains under Cu deficient conditions, though there are residual basal levels of mRNA in the Cn*cuf1Δ* strain. While CnCtr4 mRNA levels were robustly induced in response to Cu deficient conditions, we were unable to detect any Cn*CTR4* mRNA in Cn*cuf1Δ* strains (Figure 6A).

The expression of *CMT1*, *CMT2*, Cn*CTR1* and Cn*CTR4* was also analyzed and quantitated using real-time RT-PCR in the wild type, Cn*cuf1Δ* strain and the Cn*cuf1Δ* reconstituted strain (Figure 6B). The steady state mRNA levels of the four members of the *C. neoformans* Cu regulon are significantly reduced in the *cuf1Δ* strain compared with the parental wild type strain (*CMT1*, $p<0.0005$; *CMT2*, $p<0.004$; Cn*CTR1*, $p<0.0002$; Cn*CTR4*, $p<0.000002$). Consistent with phenotypic complementation analyses (Figure 5), the Cn*cuf1Δ* strain in which the wild type Cuf1 gene had been integrated into the genome restored mRNA expression levels to at or near wild type levels in cells grown under Cu limitation or excess conditions. (Figure 6B).

In *S. cerevisiae*, expression of Cu transporters or metallothioneins is directly driven by the binding of transcription factors, Mac1 or Ace1, to distinct metal regulation motifs in the promoter regions (Jungmann et al., 1993, Yamaguchi-Iwai *et al.*, 1997, Jensen et al., 1998, Thiele, 1988, Furst *et al.*, 1988, Buchman *et al.*, 1989, Labbe et al., 1997). Expression of Cu transporters from *S. pombe* is activated by Cuf1 via Cu signaling elements (CuSE), 5′ D(T/A)DDHGCTGD-3′ (D = A, G, or T; H = A, C, or T), where the GCTG motif is the essential core region for Cuf1 regulation (Beaudoin & Labbe, 2001). We searched for potential CuSE motifs in the promoter sequences from Cn*CTR1*, Cn*CTR4*, *CMT1* and *CMT2*, and identified at least one CuSE motif in the upstream sequence from each gene (Figure 6C). We also identified multiple CuSE-like motifs, which contain the exact CuSE motif with one nucleotide difference outside of the core region. Given that CnCuf1 and SpCuf1 share homology with the amino-terminal region (Jiang et al., 2011), we speculate that Cuf1 may activate expression of target genes via these potential motifs (Beaudoin *et al.*, 2003).

Our results, and data previously reported by others, indicate that Ctr4 expression is very tightly regulated by Cu availability (Waterman et al., 2007). However, in addition to Cuf1-dependent activation, we find that the expression of Cn*CTR1* is not exclusively regulated by Cu, as we observed strong basal levels of Cn*CTR1* expression by both mRNA analyses and immunoblotting (Figures 1A, 2B, 6A and 6B). This could suggest that CnCtr1 functions in Cu import under growth conditions that are not strongly Cu deficient, while Ctr4 may function under growth conditions of more extreme Cu deficiency. This would also be consistent with a growth defect observed on Cu deficient medium for the Cn*ctr1Δ* strain, but not the Cn*ctr4Δ* strain (Figure 4A). One approach to ascertain if *C. neoformans* is sensitive to the loss of either the Ctr1 or Ctr4 Cu transporters is to evaluate the levels of mRNA for one gene in the absence of the other transport gene. We used real-time PCR to measure the expression of Cn*CTR1* and Cn*CTR4* mRNAs in the isogenic wild type, Cn*ctr1Δ* and Cn*ctr4Δ* strains. As shown in Figure 5D, Cn*CTR1* mRNA levels are not influenced by the absence of the Cn*CTR4* gene under either non-Cu-stress (SC medium) or Cu deficient conditions (Figure 6D, left panel). However, expression of Cn*CTR4* is strongly increased, by over 25 fold, when Cn*CTR1* is inactivated and cells are grown under standard growth conditions. The Cn*ctr1Δ* strain grown under Cu deficient conditions exhibits no statistical significance in the levels of Ctr4 mRNA (Figure 6D, right panel). Taken together, these data suggest that under standard growth conditions *C. neoformans* senses the loss of the Ctr1 high affinity Cu transporter, but not the loss of the Ctr4 transporter, and responds by elevating expression of Ctr4.

Discussion

Studies over many decades demonstrate that the fitness of infectious agents, or their ability to survive and replicate within the host, is a crucial factor in virulence (Divon & Fluhr, 2007). As fitness is determined in part by the ability of invading pathogens to compete for limited nutrients with the host, altering the availability of nutrients that contribute to the fitness of a fungal pathogen can shift the balance to favor control by host cellular and humoral immune systems (Sexton & Howlett, 2006, Khardori, 1989). One widely accepted virulence factor for microbial pathogens is Fe and microbial Fe uptake genes have been well established to contribute to the severity of microbial infections (Almeida *et al.*, 2008, Ramanan & Wang, 2000, Jung et al., 2009, Jung et al., 2008, Nevitt & Thiele, 2011). The correlation between Fe uptake and *C. neoformans* virulence has been extensively studied. An Fe uptake regulator, CnCir1, controls the expression of genes encoding the Fe acquisition machinery both positively and negatively, and is involved in both virulence and in the mating process (Jung et al., 2008, Jung & Kronstad, 2011). Indeed, patients with the common Fe overload disease hemochromatosis, are highly susceptible to microbial infection due to the Fe-rich physiological environment that facilitates the competition for normally

limited Fe (Ashrafian, 2003, Bullen, 2000). While studies suggest that many factors contribute to the fitness and virulence of *C. neoformans*, recent reports suggest the possibility that Cu is a key virulence factor for this fungal pathogen (Waterman et al., 2007, Zhu & Williamson, 2003).

The molecular determinants involved in Cu homeostasis have been quite well characterized in non-pathogenic fungi, such as *S. cerevisiae* and *S. pombe*. In *S. cerevisiae*, Ctr1 and Ctr3 are two high affinity Cu transporters, localized on the plasma membrane, that transport extracellular Cu into cells where it is distributed for utilization by a dedicated set of Cu chaperones, assembly factors and pumps (Pena *et al.*, 2000). The expression of both Cu transporters is positively controlled by the Mac1 transcription factor which under low Cu conditions is bound to Cu responsive elements (CuREs) in these and other promoters (Jungmann et al., 1993, Yamaguchi-Iwai et al., 1997, Jensen et al., 1998, Labbe et al., 1997). The *S. cerevisiae* genome also encodes another Cu transporter, Ctr2, which localizes on the vacuole membrane and, in concert with the Fre6 metalloreductase, transports Cu from the vacuolar lumen into cytosol under conditions of external Cu deficiency (Rees et al., 2004, Rees & Thiele, 2007). Similar to the expression of the homo-trimeric *CTR1* and *CTR3* genes under low Cu conditions, the expression of *S. pombe CTR4* and *CTR5* genes, which encode hetero-trimeric Cu transporter subunits, is regulated by the *S. pombe* Cuf1 Cu metalloregulatory transcription factor (Beaudoin & Labbe, 2001, Beaudoin *et al.*, 2006, Beaudoin *et al.*, 2011, Zhou & Thiele, 2001, Labbe *et al.*, 1999). While Mac1 directly activates transcription of the Cu uptake machinery, the *CUP1* and *CRS5* metallothionein genes, encoding critical components of the Cu detoxification machinery, are directly activated by the Cu-activated transcription factor Ace1 (Pena et al., 1998, Thiele, 1988, Culotta et al., 1994).

Many organisms, such as plants, fungi, and flies, encode multiple Cu transporters of the Ctr family in the genome (Yuan *et al.*, 2011, Penarrubia *et al.*, 2010, Puig & Thiele, 2002, Zhou *et al.*, 2003). Here we demonstrate that *C. neoformans* genome encodes two functional Cu transporters in this family. One gene, *CNAG_07701*, encodes a previously uncharacterized Cu transporter in *C. neoformans*. Recently, one report suggested that *CNAG_07701* encodes a vacuolar Cu transporter, Ctr2, based on computational predicted protein sequence (Chun & Madhani, 2010). However, our cDNA sequence from *CNAG_07701* is not orthologous to Ctr2 (Figure 3 and supplemental files 3, Figure S1&2). We deciphered the evolutionary relationship of *CNAG_07701* using phylogenetic analysis and we demonstrate that the protein sequence encoded from the *CNAG_07701* cDNA is homologous to Ctr1 in many other fungal species. Expression of the cDNA sequence from *CNAG_07701* in Sc*ctr1Δ/ctr3Δ* cells compensates for the Cu importing function of Sc*CTR1* and Sc*CTR3*, indicating that *CNAG_07701* encodes a Cu importer. Similar to Cu importers from other organisms (Pena et al., 2000, Puig *et al.*, 2002), the protein encoded by *CNAG_07701* localized at least partially to the plasma membrane. Considering the sequence, functional and regulation evidence, we have renamed *CNAG_07701* as Cn*CTR1*. While both Cn*CTR1* and Cn*CTR4* have independent roles in Cu transport, we observed that Cn*CTR1* may play a dominant function in Cu acquisition under standard laboratory growth conditions and the absence of expression of Cn*CTR1* significantly elevates that of Cn*CTR4* under these same conditions.

In this work, deletion of Cn*CTR1* alone strongly affects cell growth under low Cu conditions, in agreement with the results by Chun and Madhani (Chun & Madhani, 2010). However, we did not observe defects in melanin production or capsule formation in the Cn*ctr1Δ* strain, even in the presence of BCS (data not shown). This may be due to the difference of laboratory strain backgrounds. It has been previously demonstrated that the expression of *CTR4* is species-dependent in *S. cerevisiae* and *C. neoformans* (Knight et al., 1996, Waterman et al., 2007). A transposable element was first described in *S. cerevisiae*

within the promoter sequence of *CTR3* (Knight et al., 1996), which masks the expression of the *CTR3* Cu transporter in some strains. A similar feature may also be conserved in *C. neoformans*. Waterman and colleagues demonstrated that expression of *CTR4* varies dramatically among clinical isolates (Waterman et al., 2007). We identified many *CTR4* promoter homologous sequences in chromosomes 1, 3, 7, 8, 13 and 11 that could represent *C. neoformans* transposon-like elements (http://www.broadinstitute.org/annotation/genome/cryptococcus_neoformans/Blast.html).

In this report we demonstrate the surprising observation that the *C. neoformans* Cu metalloregulatory transcription factor Cuf1 is important for activating expression of both the Cu acquisition machinery and the Cu detoxifying metallothionein genes. Consistent with this observation, Lin and colleagues demonstrated that a *cuf1Δ* mutant in *C. neoformans* serotype D exhibited a growth defect on both low and high Cu medium and Jiang and colleagues also demonstrated a Cu sensitive phenotype in a *cuf1Δ* strain in *C. neoformans* serotype A (Jiang et al., 2011, Lin et al., 2006). However, as Cu can be toxic due to the generation of reactive oxygen species, through the inhibition of Fe-S cluster formation and other mechanisms (Chillappagari *et al.*, 2010, De Freitas *et al.*, 2000, Liochev, 1996, Macomber & Imlay, 2009, Macomber *et al.*, 2007), the manner by which Cuf1 protects *C. neoformans* from Cu toxicity were not clear. Our observation that Cuf1 is also important for the Cudependent expression of *CMT1* and *CMT2* mRNAs would, at least in large part, explain this duality of function. Based on sequence similarity Mac1 and Ace1 are paralogs that have likely obtained different functions through sequence divergence. The dual regulation of Cu transporter and metallothionein genes was previously observed in *Drosophila melanogaster*, in which the MTF-1 transcription factor was demonstrated to directly participate, via promoter Metal Regulatory Element binding, in the activation of the *CTR1B* gene and four genes encoding metallothioneins (Selvaraj *et al.*, 2005, Egli *et al.*, 2003, Zhou et al., 2003). The mechanisms by which the *C. neoformans* Cuf1 protein functions to activate the expression of distinct genes under Cu deficiency or Cu excess conditions is currently under investigation.

In this work we elucidate the transcript profile of *C. neoformans* in response to Cu deficiency and Cu excess conditions. These studies indicate that Cu deficiency conditions elevate the levels of transcripts encoding multiple plasma membrane high affinity Cu importers, as well as transcripts encoding other proteins whose functions must be experimentally evaluated. Moreover, we demonstrate that CnCuf1 activates expression of the Cu detoxifying metallothioneins and additional genes that may facilitate adaptive responses to high Cu. The activation of expression of Cu transporters and metallothioneins may be through the binding of Cuf1 to CuSE motifs and we are currently investigating the recognition site for Cuf1. Previous studies demonstrated that a *C. neoformans cuf1Δ* mutant has strongly attenuated virulence in mouse tail vein infection models and that *C. neoformans* strains isolated from patient's brain tissue tend to express elevated levels of Ctr4 (Waterman et al., 2007). While these studies suggested that Cu acquisition is a virulence factor for *C. neoformans*, our discovery of a dual role for Cuf1 in gene expression of both the Cu acquisition and detoxification pathways calls for a thorough evaluation of the specific Cuf1 target genes required for virulence and their mechanisms of action.

Material and Methods

Strains and media

Cryptococcus neoformans strains (Supplemental file 1, Table S1) were routinely grown in YPD medium (1% yeast extract, 2% peptone, 2% dextrose) at 30°C. Synthetic complete medium (114400022, MP Biomedicals, Inc.) was used for RNA extraction for cDNA synthesis and RNA blots. YPD supplemented with 1.5% agar and 100 μg/ml of

nourseothricin, 200 μg/ml of neomycin, or 200 U/ml hygromycin B was used for colony selection after biolistic transformation. SCEG agar (synthetic complete medium supplemented with 1.5% agar, 2% ethanol and 3% glycerol) was used to test the cell growth of Cu transporter mutants. L-DOPA plates were used for melanin production assay, as previously described (Chaskes & Tyndall, 1978).

RNA isolation

Cells that were grown in 5 ml YPD medium at 30°C for 24 hr were collected and washed three times with sterile water. Cells were then diluted in fresh SC medium to an A_{600} of 0.2, and the culture was incubated at 37°C for 3 hr to allow cells to recover from the lag phase. After 3 hr incubation, $CuSO_4$ or bathocuproin sulfonate (BCS) was added, and the cell cultures were kept at 37°C until being removed at specific time points for RNA isolation. RNA was isolated using the phenol-chloroform method. The genomic DNA was eliminated using TURBO DNA-*free* (Ambion, inc.). The quality of RNA was confirmed by measuring the 260/280 ratio on a spectrophotometer and visualizing on RNA gels. For RNA blots, DNase I treated RNA was separated, transferred, and hybridized. The probes were amplified from cDNA using oligonucleotide pairs ACT1F/R (actin), CTR1F/R (Cn*CTR1*), CTR4F/R (Cn*CTR4*), MT1F/R (*CMT1*), and MT2F/R (*CMT2*). The DNA probes were labeled with ^{32}P and mRNA detected and quantified using a phosphorimager (Amersham, inc.).

C. neoformans cDNA sequencing and complementation in *S. cerevisiae*

cDNA sequences of *CMT1*, *CMT2*, Cn*CTR1* and Cn*CTR4* were obtained using FirstChoice@ RLM-RACE kit (Ambion, Inc.). The RNA samples were processed as described in the manufacturer's manual, except cDNA was reverse transcribed using Superscript III@ First-Strand Synthesis system (Invitrogen, Inc.). PCR products were generated using *Taq* platinum and were cloned using a TA cloning kit (Invitrogen, Inc.). The cloned plasmids were sequenced by the Duke University DNA analysis facility. Multiple clones were sequenced for each cloning, and each gene was repeated twice using independent RNA samples (Supplemental file 2).

cDNA sequences encoding *CMT1*, *CMT2*, Cn*CTR1* and Cn*CTR4* were amplified using oligonucleotide pairs MT1expF/MT1expR, MT2expF/MT2expR and CTR1expF/CTR1expR, CTR4expF/CTR4expR respectively (Supplemental file 1, Table S2). The amplified cDNA sequences were digested with restriction enzymes *EcoR*I and *Hind*III, and cloned in *S. cerevisiae* expression vector p426GPD. The expression of cloned cDNA is driven by a GPD promoter. The Sc*cup1Δ* strains were transformed with vectors containing the cDNA sequence from *CMT1* or *CMT2*, and The Sc*ctr1Δ/ctr3Δ* strain was transformed with vector containing cDNA sequence from Cn*CTR1* or Cn*CTR4*. Strains that were transformed with p426GPD vector were used as controls. To test the complementation by metallothionein proteins or Cu transporters, transformed cells were spotted on SC agar supplemented with 200 μm Cu or SCEG agar, respectively.

cDNA synthesis for transcript profile analysis

The DNA microarray slides for *C. neoformans* were purchased from the Washington University Genome Center, representing the whole genome of serotype A and D. Each slide contains 11,343 oligo probes, printed in duplicate. The DNA microarray slides and RNA samples were submitted to Duke Genome Center and were processed by the microarray core facility. For cDNA synthesis, RNA was isolated as described, and was sequentially cleaned using RNeasy kit (Qiagen, Inc.). The quality of RNA was confirmed using a bioanalyzer (Agilent, Inc.). RNA samples were then first amplified using MessageAmpII aRNA kit (Ambion, Inc.), and sequentially labeled with Cy3 or Cy5 dye. The microarray experiment was performed using three independent isolated RNA samples, including one dye swap

labeling. Microarray slides were hybridized, washed and scanned using Axon Genepin Scanner.

Real-time PCR

RNA samples were isolated as above. 1 μg total RNA was reverse transcribed to cDNA. The real-time PCR was performed using iQ™ SYBR Green Supermix on a Biorad iQ™ 5 real-time PCR detect system (Biorad, Inc.). The amplification conditions consist of denaturing step at 95°C for 3 min, 45 cycles of 95°C for 10 s and 60°C for 45 s, 95°C for 1 min, 55°C for 1 min, 81 cycles of 55°C for 10 s, and PCR reaction was stored at 20°C. The results were normalized to Cn*ACT1*, and analysis using $2^{-\Delta\Delta Ct}$ method as previously described (Rossignol *et al.*, 2009). Statistical significant was calculated using Student's t-test.

Microarray annotation and data analysis

Cryptococcus version 2 microarray probe sequences were downloaded from the manufacturer's website in Washington University (http://gtac.wustl.edu/). Because this microarray was originally designed based on *Cryptococcus neoformans* JEC21 genome, the probes was remapped to *C. neoformans* H99 genome in this study. *C. neoformans* H99 genome sequence was downloaded from Broad Institute (http://www.broadinstitute.org/annotation/genome/cryptococcus_neoformans/MultiHome.html). Probe was remapped based on the protocol previously described (Yin *et al.*, 2010). The 70-mer probe sequences were aligned to the genome from H99 using BLASTN (Altschul *et al.*, 1990). Probes, matched the genome uniquely with ≥ 65bp, were retained for further analysis. Probes, match several targets in the genome or without any match to the genome, was excluded to minimize possible unspecific signals. The genome specific probes were mapped to the annotated genes on H99 genome. Probes, not matching any annotated genes, was excluded from the analysis.

Microarray signal intensities were normalized and summarized using Bioconductor package limma (Smyth, 2004). Probes with low signals were excluded from statistical analysis. Differentially expressed genes were selected using Bioconductor package samr (Tusher *et al.*, 2001) with cut-off of Qvalue<0.05 and fold change of ≥1.5 or ≤0.67. For genes with multiple probes, splicing Index was calculated as previously described (Yin et al., 2010). Genes with splicing Index ≥1 or ≤−1, were predicted as alternative spliced. The probes were used separately to indicate transcript level expression. If the splicing Index was below this threshold, the fold changes for all probes were averaged to reveal gene level expression.

Sequence data and database searches

Our fungal protein database consisted of 102 genomes (Supplemental file 1, Table S3). Where available, data was obtained from the NCBI fungal genome FTP site (ftp://ftp.ncbi.nih.gov/genomes/Fungi). The remaining data was downloaded from the relevant sequencing centres (Supplemental file 1, Table S3).

Saccharomyces cerevisiae amino acid sequences were obtained for Ctr1 (YPR124W), Ctr2 (YHR175W) and Ctr3 (YLR411W) from the *Saccharomyces* Genome Database (http://www.yeastgenome.org/). These along with the amino acid sequence of *CNAG_07701* were used as bait in our database search. Using the HMMER package (http://hmmer.org/) we scored the presence or absence of these 4 proteins in each fungal proteome used in this analysis. A bidirectional database search with a cutoff E-value = 10^{-5} was performed. We consider proteins located by this bidirectional strategy as orthologs and noted these. Proteins located in a one direction hit were initially considered as homologs.

Phylogenetic methods

Our database searches located 332 Ctr proteins. These were extracted and aligned using MUSCLE (v3.6) (Edgar, 2004), with the default settings. Obvious alignment ambiguities were manually corrected. Phylogenetic relationships of all 332 Ctr proteins were constructed using the Neighbor-joining method implemented in the software Quicktree (Howe *et al.*, 2002). One hundred bootstrap replicates were performed and summarized using the majority-rule consensus method.

A maximum likelihood phylogeny was also inferred for a representative dataset. The appropriate protein models of substitution were selected for each gene family using ModelGenerator (Keane *et al.*, 2006). One hundred bootstrap replicates were then carried out with the appropriate protein model using the software program PHYML (v3.0) (Guindon & Gascuel, 2003) and summarized using the majority-rule consensus method.

Generation of mutants

C. neoformans knockout cassettes were generated using the overlapping PCR method (Davidson *et al.*, 2002), and transformation was performed using biolistic system as previous described (Toffaletti *et al.*, 1993). Briefly, upstream sequence from *CTR1*, *CTR4*, *CMT1*, *CMT2, or CUF1* was amplified using oligonucleotide pairs CTR1UPF/R, CTR4UPF/R, MT1UPF/R, MT2UPF/R, or CUF1UPF/R, respectively (Supplemental file 1, Table S2). Downstream sequence from *CTR1*, *CTR4*, *CMT1*, *CMT2* or *CUF1* was amplified using oligonucleotide pairs CTR1DWF/R, CTR4DWF/R, MT1DWF/R, MT2DWF/R, or CUF1DWF/R respectively. Selective markers were amplified from plasmids pJAF1 or pAI3 using oligonucleotide pairs M13F/R, respectively. A PCR knockout cassette was generated by combining upstream and downstream sequences from target gene with selective marker. The PCR product was then purified, concentrated and transformed into cells using biolistic. Potential transformants were tested for transformation stability. Southern blotting and PCR were performed to confirm the presence of the selective marker and the absence of deleted fragment (data not shown). RNA blot or reverse transcriptase PCR was performed to confirm the loss of mRNA (data not shown).

For *CUF1* complementation, wild type *CUF1* was amplified using the oligonucleotide pairs CUF1REF/R, and the product was digested and cloned at restriction site *Sac*I in plasmid pHYG7-KB1 (a gift from Dr. Jennifer Lodge, Washington University) (Hua *et al.*, 2000). The resulting plasmid was then transformed into a Cn*cuf1Δ* strain. The complementation of wild type Cuf1 in the Cn*cuf1Δ* strain was confirmed using spotting assays and real-time PCR.

Flag-CTR1 and *CTR1-Flag* were generated using overlap PCR. For *Flag-CTR1*, genomic DNA sequence was amplified using oligonucleotide pairs CTR1_1R/CTR1SACIF or CTR1_1F/CTR1SACIR, introducing DNA sequence encoding 1 X flag protein at the 5′ of *CTR1*. Two PCR products were used to generate an overlap PCR product, which was then cloned at *Sac*I site in plasmid, pHYG7-KB1. The resulting plasmid was transformed into a Cn*ctr1Δ* strain. The expression of FLAG tagged Cn*CTR1* was confirmed by western blot and phenotype complementation on SC agar supplemented with 1 mM BCS (Figure 2B and 5A). The same method was applied for generating *CTR1-Flag*, except oligonucleotide pairs CTR1SACIF/CTR1_5R or CTR1SACIR/CTR1_5F was used for PCR, introducing Flag DNA sequence at 3′ of *CTR1.*

C. neoformans immunoflourescent microscopy

The immunoflourescent microscopy was performed as described previously (Liu *et al.*, 2006). Overnight yeast cultures were diluted in 10 ml of SC medium at A_{600} 0.2. After 3 hr

incubation at 37°C, BCS was added to a final concentration of 1 mM to induce the expression of *CTR1-Flag* or *Flag-CTR1* for additional 3 hr. Cells were then fixed with formaldehyde, washed with PBS, and resuspended in sorbitol-containing buffer. Lysing enzyme (L1214, Sigma, Inc.) supplemented with protease inhibitor (Roche, Inc.) was used to generate spheroplast. We noticed that the addition of protease inhibitor enhance the signal. Cells were then washed with PBS and resuspended in PBS supplemented with BSA. Cells were attached to polylysine treated glass slide, and incubated with anti-FLAG antibody at 4°C overnight, and washed with PBS + BSA. Secondary antibody (conjugated Alexa flour 488) was added and incubated at room temperature for 30 min. The slides were washed, mounted, and observed using a Zeiss Axio Imager Microscopy.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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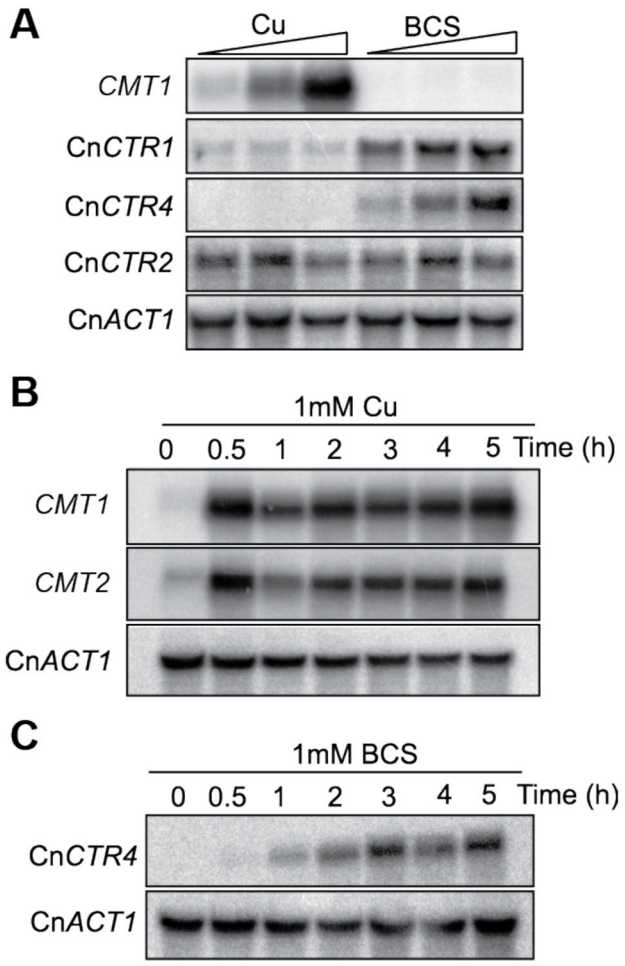

Figure 1. Identification of Cu regulons in *C. neoformans*

A. *C. neoformans* H99 was treated with 10 μM, 100 μM, or 1 mM $CuSO_4$ or BCS for 3 hr at 37°C. RNA samples were hybridized with radioactively labeled DNA probes corresponding to the *CMT1*, Cn*CTR1*, Cn*CTR4*, or Cn*ACT1* open reading frames.

B. H99 cells were treated with 1 mM $CuSO_4$. RNA samples were isolated from six time points (0, 30 min, 1, 2, 3, 4, or 5 hr). The RNA blot was hybridized with radioactively labeled DNA probes from *CMT1*, *CMT2* or Cn*ACT1*.

C. H99 cells were treated with 1 mM BCS. RNA samples were isolated as described in B and The RNA blot was hybridized with DNA probes from Cn*CTR4* or Cn*ACT1*.

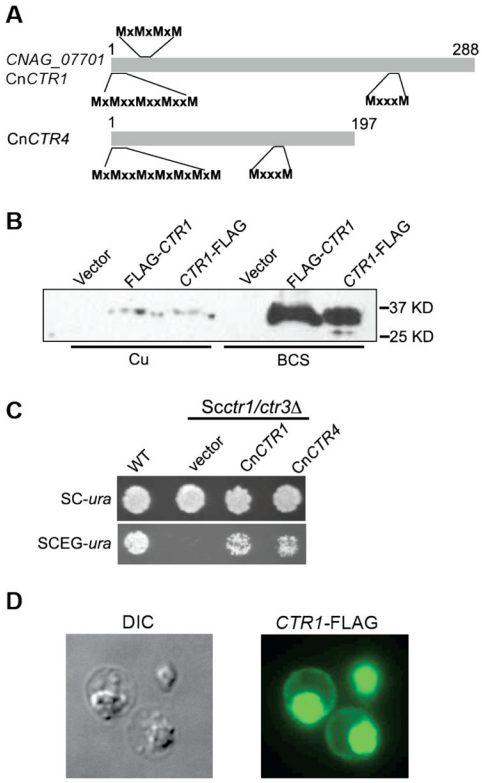

Figure 2. Structural features and functional complementation by *C. neoformans* Cu transporters

A. Scheme of protein sequences from Cn*CTR1* and Cn*CTR4*. The protein sequences from Cn*CTR1* and Cn*CTR4*, the first and last resides were labeled with numbers. Approximate locations of methionine-rich motifs were labeled with MxM.

B. The protein from Cn*CTR1* was tagged with Flag epitope at the amino- or carboxyl-terminus. Cells were grown at 37°C for 3 hr in SC medium supplemented 1 mM Cu or 1 mM BCS. Protein was isolated and quantified using Biorad protein assay. Equal amount of protein was loaded for each strain. Proteins were visualized by Ponceau S staining after semi-dry protein transfer to confirm the protein loading (data not shown). Western blot was then performed using FLAG antibody.

C. cDNA sequences from Cn*CTR1* and Cn*CTR4* were transformed into Sc*ctr1Δ/ctr3Δ* strain. The complementation of cDNA from *C. neoformans* was examined by growing transformed *S. cerevisiae* cells on SC-*ura* or SCEG-*ura* agar at 30°C for 3 days.

D. Cells expressing Ctr1-Flag protein were grown in SC medium supplemented with BCS for 3 hr. The localization of Flag epitope was visualized by immunofluorescence microscopy as described in Material and Methods. *DIC,* differential interference microscopy.

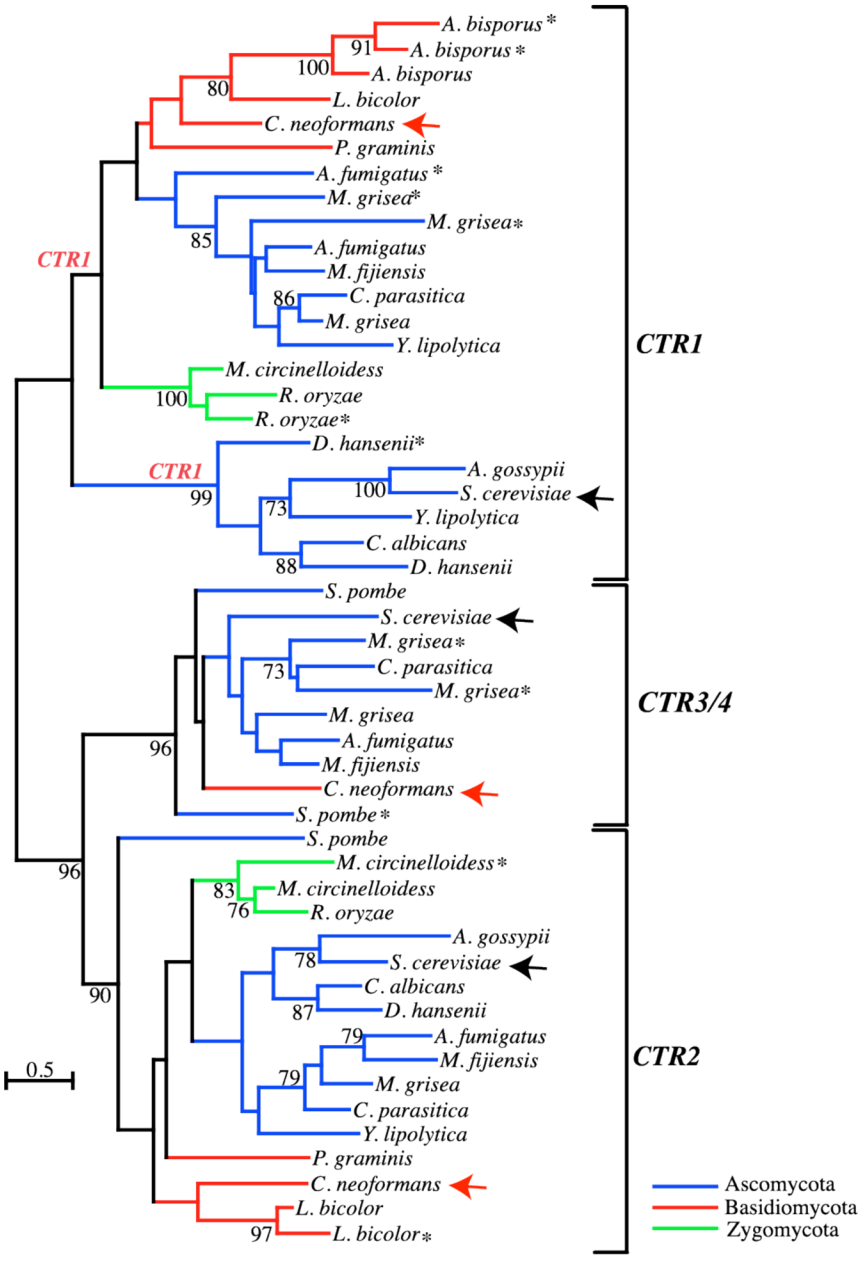

Figure 3. Maximum likelihood Ctr representative phylogeny

Bootstrap resampling (100 iterations) was undertaken. Only branches with greater than 70% bootstrap support are labelled. Branches are colored according to their taxonomy. Monophyletic clades for Ctr1, Ctr2 and Ctr3 are labelled. Species names followed by an asterix indicate that the underlying protein is a paralog (based on a one direction database hit). Ctr proteins from *C. neoformans* are labelled with red arrows, and that from *S. cerevisiae* are labelled with black arrows.

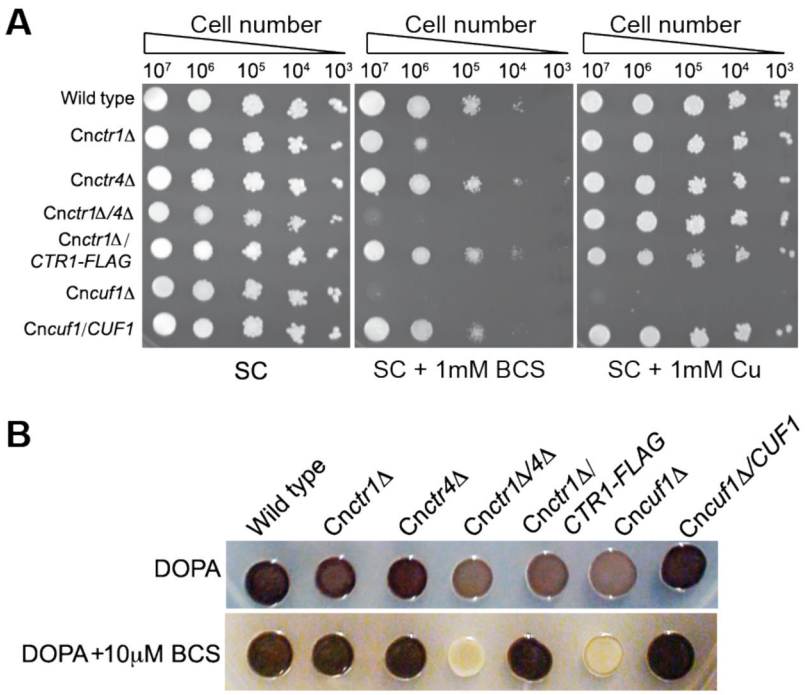

Figure 4. Gene disruption of Cu transporters in *C. neoformans*

A. Cell cultures were diluted with water to A_{600} of 1, 0.1, 0.01, 0.001, or 0.0001 and five microliters of four cell suspensions were spotted on SC agar or SC agar supplemented with 1 mM BCS or 1 mM $CuSO_4$. The plates were incubated at 37°C for 3 days.

B. DOPA agar plates were used for melanin production assays. Cell suspensions were prepared as described in A and cell suspensions were spotted onto DOPA agar or DOPA agar supplemented with BCS. Plates were incubated in the dark at 37°C for 2 to 4 days. Plates were examined and photographed for pigment development every day. Only the first row (initial A_{600} of 1.0) from each plate is shown.

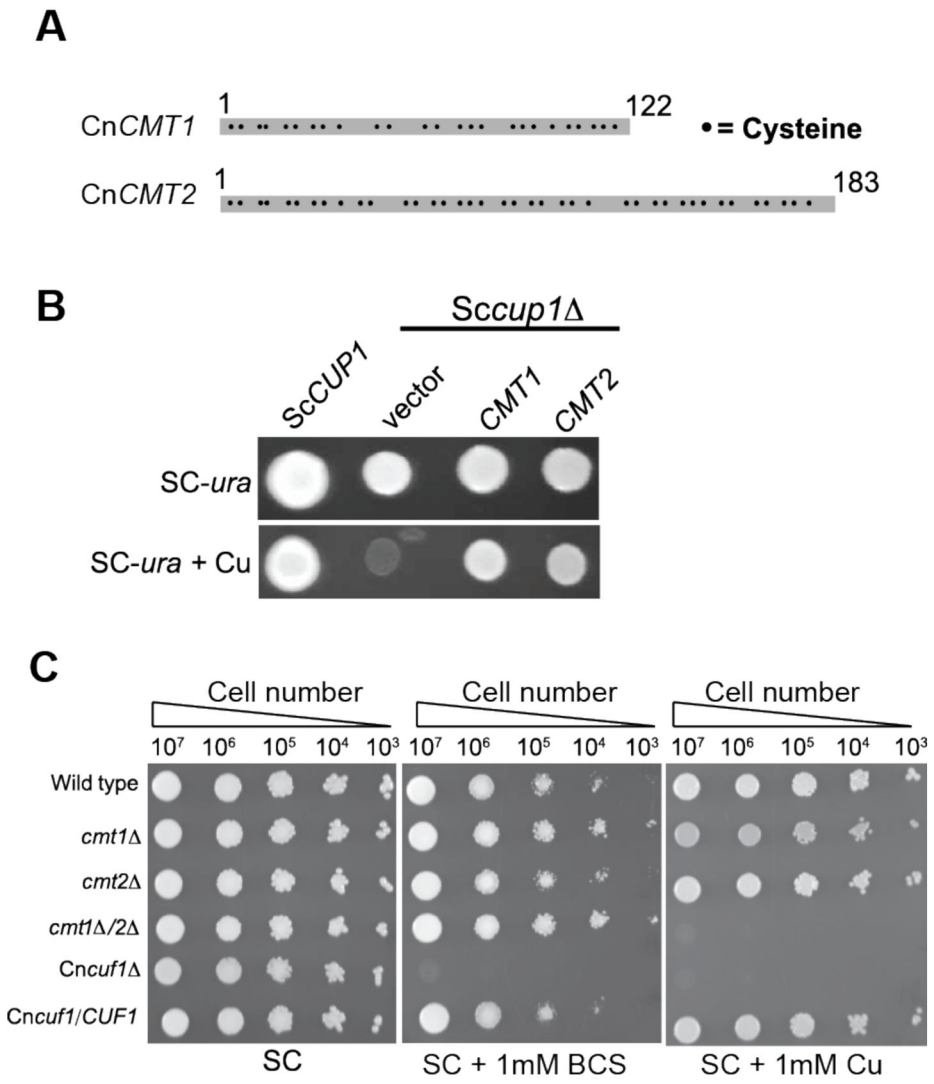

Figure 5. Characterization of metallothioneins in *C. neoformans*

A. Scheme of protein sequences from Cmt1 and Cmt2, the first and last residues were labeled with numbers. Approximate locations of cysteine residues on each protein were labeled with black dots.

B. cDNA sequences from *CMT1* and *CMT2* were transformed into Sc*cup1Δ*. The complementation was examined by growing transformed cells on SC or SC agar supplemented with 200 μm $CuSO_4$ at 30°C for 3 or 4 days.

C. The experiment was carried out exactly the same as described in Figure 4A.

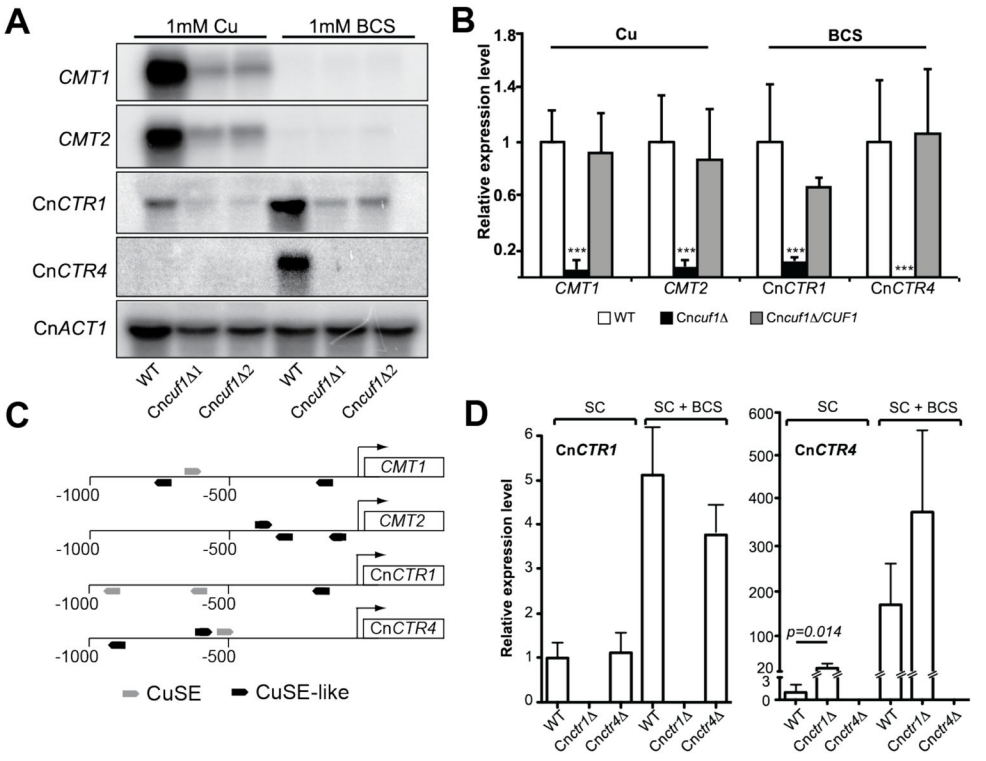

Figure 6. Regulation of metallothionein and Cu transporter mRNA levels

A. The wild type and two independent Cn*cuf1Δ* strains were pre-incubated in SC medium at 37°C. After 3 hr incubation, 1 mM BCS or 1 mM $CuSO_4$ was added, and cell cultures were kept at the same condition for additional 3 hr. RNA samples were isolated, and RNA blot was hybridized with DNA probes from each target.

B. The wild type, Cn*cuf1Δ* and *CUF1* complementation strains were grown exactly the same as described in A. RNA was isolated, DNase treated, and reverse transcribed to cDNA. Real-time PCR was then performed. The experiment was carried out using five independent biological replicates. Student's t-test was performed to determine statistical significant. Significant changes in expression is indicated by asterisk, where triple asterisks represent $p<0.001$.

C. Possible CuSE motifs were mapped within the promoter regions of Cu transporters and metallothioneins. A 1 kb DNA sequence from each gene was isolated. Motifs with CuSE 5′-D(T/A)DDHGCTGD-3′ (D = A, G, or T; H = A, C, or T) sequence were labeled with grey box. Motifs similar to CuSE sequence with one nucleotide difference in non-core region were labeled with black box. The orientation of motifs is shown by arrows.

D. The wild type, Cn*ctr1Δ* and Cn*ctr4Δ* strains were grown the same as described in A. The real-time PCR and analysis was performed as described in B.

Table 1

Genes differentially expressed in cells treated with Cu or BCS for 3 hr.[a]

Conditions	Gene ID	LogFC	Description
High Cu	CNA G_05449	−4.56	Metallothionein
	CNAG_00306	−3.55	Metallothionein
	CNAG_07840	−2.89	Unknown function
	CNAG_02427	−1.82	Isochorismatase
	CNAG_02691	−1.43	Unknown function
	CNAG_04358	−1.36	Iron-sulfur clusters transporter
	CNAG_01102	−1.34	Oxidoreductase
	CNAG_06424	−1.04	Claudin family protein
	CNAG_03408	−1.02	Unknown function
	CNAG_06668	−0.97	Mitochondrial protein
	CNAG_00663	−0.97	Unknown function
	CNAG_02933	−0.90	Quinone oxidoreductase
	CNAG_00834	−0.71	Phosphatidylserine decarboxylase
	CNAG_01255	−0.60	Unknown function
Low Cu	CNAG_06208	0.73	Heat shock protein
	CNAG_06205	0.75	Unknown function
	CNAG_00110	0.79	Rho GTPase binding
	CNAG_02864	1.51	Unknown function
	CNAG_07701	2.00	Cu transporter
	CNAG_04387	2.09	Pre-mRNA splicing factor
	CNAG_00876	2.87	Ferric-chelate reductase
	CNAG_02775	3.61	Unknown function
	CNAG_00979	5.06	Cu transporter

[a]Cells at exponential phase were treated with 1 mM $CuSO_4$ or 1 mM BCS for 3 hr at 37°C. RNA samples were isolated and transcript profiles were compared between cells treated with Cu and BCS. Each experiment had three biological replicates. FC, fold change.