The Cell Biology of Virulence – Lessons from the Pathogenic Fungus *Cryptococcus neoformans*

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Cryptococcus neoformans is a yeast-like fungus that is a major cause of morbidity and mortality in patients with cell-mediated immune defects. Pathogenic strains of *C. neoformans* are thermotolerant, growing well at mammalian host temperature, and produce a large polysaccharide capsule that is antiphagocytic and immunomodulatory. Also correlated with pathogenicity is the ability to produce melanin pigments, a trait that results from expression of the multi-copper oxidase, laccase. Since the laccase of *C. neoformans* was purified and its corresponding gene identified, much effort has been placed on understanding factors that affect the production of laccase at the level of transcription, posttranslational modification and trafficking. Our study of laccase has led to the development of tools with which to study the expression of processes essential to cryptococcal virulence, allowing cell biology to inform our understanding of cryptococcal pathogenesis. In this report we detail the development of tools for the study of the cell biology of *C. neoformans* during mammalian infection, and how these tools are being used to investigate the virulence of this important fungal pathogen.

Keywords virulence; cell biology; neurotropism; trafficking; clinical diversity

1. *Cryptococcus* and cryptococcosis

The yeast-like fungus *Cryptococcus neoformans* causes an often-fatal meningo-encephalitis. Although infections in patients with intact immune systems do occur, the infection is most often seen in patients with immune defects [1]. Cell-mediated immunity, comprised mainly of $CD4^+$ helper T lymphocytes and $CD8^+$ cytotoxic T lymphocytes, are the main line of defense against *Cryptococcus* infection. There are two major groups of patients that are at high-risk for acquiring an infection by *C. neoformans*: 1) Those that are immunosuppressed by medical intervention, and 2) those that are immunosuppressed by a confounding medical condition. Medical interventions that predispose to cryptococcal infection include chemotherapy in cancer patients, and immunosuppressive drugs to prevent graft rejection or graft-verus-host disease in transplant recipients. Confounding medical conditions include hematologic malignancies, such as leukemia or lymphoma, and the development of AIDS as a result of HIV infection. In developed countries, infections by *C. neoformans* continue to be a significant source of medical complications of transplant recipients, increasing the time of hospitalization and the cost of care [2]. In regions of the world where HIV infection rates continue to rise, *C. neoformans* is a major cause of mortality [3].

Cryptococcosis is thought to be acquired from inhalation of the infectious form, most likely a sexual or asexual spore [1]. It is unclear if cryptococcosis arises from reactivation of latent infection or from a newly acquired infection, but data from an HIV-infected cohort from France seems to suggest that reactivation may occur [4]. Patients can also develop a pneumonitis which may be subclinical or accompainied by symptoms [1]. Through hematogenous dissemination, *C. neforomans* is able to cross the blood-brain barrier and enter the central nervous system (CNS) where it causes severe inflammation and abcesses within the neural parenchyma. Each of these infectious transitions are accompanied by opposing pathogen and host responses, the outcome of which determines whether successful infection occurs and its outcome.

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There are three phenotypic characteristics common to all pathogenic strains of *C. neoformans*. These include the ability to grow at host temperature (37-39^{o}C), the production of a polysaccharide capsule, and the ability to oxidize neuro-catecholamines through the activity of the enzyme laccase. In addition, several enzymatic activities have been shown to impact virulence, including urease and phospholipase B [5, 6]. Many genes and pathways have been described that regulate the expression of these virulence traits *in vitro* and impact virulence in animal models of cryptococcosis. The challenge for the future is to investigate the function and modulation of virulence factors and the pathways that regulate them within the context of the host.

The use of murine models of cryptococcosis has provided a platform on which the pathogenesis of the infection can be investigated. The two main models in use mimic the two principal forms of cryptococcal infection. The disseminated model uses intravenous injection of outbred strains such as NIH Swiss Albino mice, allowing the ability of *C. neoformans* to enter the CNS and cause meningo-encephalitis to be assessed. In the pulmonary model, intranasal inoculation of anesthetized strains such as CBA/Jcr mice can be used to assess the ability of *C. neoformans* to survive in the lung and establish infection.

2. A Need for the Right Tools

Many of the molecular advances in the study of *C. neoformans* regarding signal pathways and effectors involved in virulence trait regulation have been achieved by *in vitro* study. Animal models have been a facile method useful to confirm the role of a given gene in overall virulence or assess an immunological response to a genetically modified strain. However, by investigating cellular events of the pathogen during 'real time' adaptation to the mammalian host, we may be able to obtain information regarding how these identified pathways function during infection and how pathogens respond to the host within different tissues. The following are two examples of investigations seeking such understanding of the cell biology of the pathogen in the context of the model host.

2.1 A role for laccase in the pathogenesis of cryptococcosis.

The ability of *C. neoformans* to produce melanin pigments in vitro has long been associated with virulence [7]. Melanin pigments are formed on the surface of *C. neoformans* yeast cells by the activity of the laccase enzyme on exogenous catecholamines (Fig 1). Initial characterizations of laccase relied on studies conducted in vitro or on isolated enzyme. For example, purified laccase was found to be a multi-copper oxidase that has a substrate preference for diphenolic compounds including host-borne substrates such as epinephrine and norepinephrine. The tissue localization of these compounds within the CNS has been hypothesized to contribute to the apparent neurotropism of *C. neoformans*. In addition, laccase was found to be repressed strongly by glucose in vitro, with transcription induced to a maximum after 3 hours of glucose starvation at 30^{o}C. Furthermore, knockout experiments demonstrated that laccase is important in central nervous system (CNS) dissemination but dispensable for survival in the lung [8]. These findings led to questions about when and where laccase is expressed during mammalian infection and whether host control of cryptococcal processes could help to explain the differential role of this important virulence factor in neurotropism of the fungal pathogen. To help answer these questions, cell biological tools needed to be developed that could analyse these fungal processes as they occurred in mammalian host models.

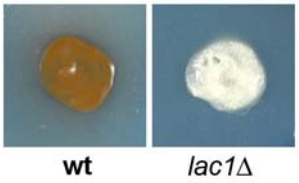

Fig. 1 Cells of wild type *C. neoformans* and the *lac1*Δ mutant incubated on norepinephrine agar at 30^{o}C for 24 hours. Cells of the wild type form dark melanin pigments whereas cells of the *lac1*Δ strain remain non-pigmented.

3. Filling the Tool-box

3.1 Vectors for episomal expression in *C. neoformans*

The ability to express recombinant DNA from an extra-chromosomal plasmid vector is a useful tool in cell biological analysis. There are many applications for episomal expression, including overexpression, RNA interference [9], protein localization by expression of fluorescent protein fusions [10] and expression of dominant negative or active mutants [11]. A series of episomal plasmid vectors has been constructed for use in *C. neoformans*. These vectors are derivatives of the pCnTel vector originally produced by J. Edman [12], and contain a *URA5* selectable marker, a telomere cassette that contains the Kan^R gene for bacterial transformation, a pBluescript origin of replication and a STAB sequence from *E. coli* that was found to promote plasmid stability in *C. neoformans* [13, 14]. A basic shuttle vector, pORA-2XK, is depicted in Fig. 2. Digestion of the plasmids with I-*Sce1* exposes telomeric repeats at each end of the linear construct that allow the episome to be maintained in *C. neoformans* as a mini-chromosome. Other versions of this vector have been constructed to contain constitutive promoters such as P_{ACT1} and P_{GPD1} as well as fluorescent proteins for reporter constructs and expression of fusion protein for localization studies. Copy number of transformed plasmids has been estimated by uncut southern blot of *C. neoformans* DNA, by comparing hybridization intensities between genomic DNA and the episome. By choosing transformants with similar relative copy number, comparisons can be made between strains harboring the same plasmid.

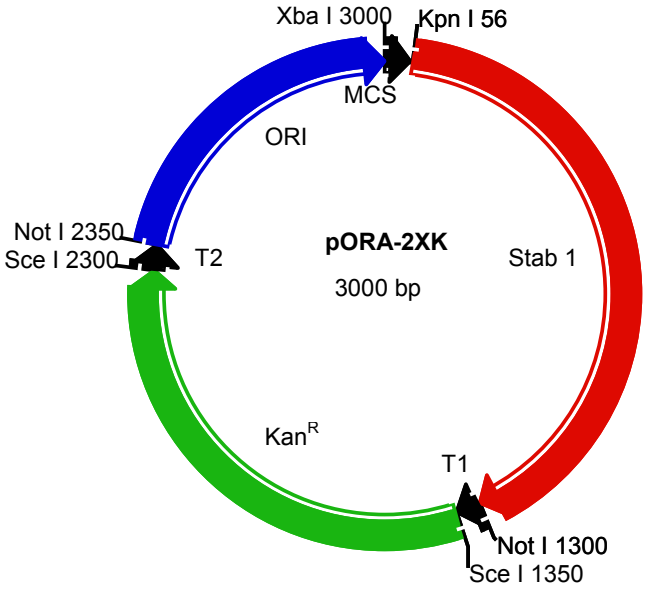

Fig. 2 Episomal vector pORA-2XK for use in *C. neoformans*. The vector contains a kanamycin resistance cassette (Kan^R), origin of replication for *E. coli* (ORI), a multiple cloning site (MCS) and a *C. neoformans* stabilization sequence (Stab1)

3.2 *In vivo* Selection

While the utility of episomal expression has been well documented from studies in model systems, the question remains as to whether an episomal vector could allow study of pathogenesis *in vivo*. It is known that *ura5*$^-$ strains of *C. neoformans* are compromised in virulence, so we hypothesized that the host environment would select for maintenance of the episome. To test this, the virulence of two independently derived strains carrying the empty expression vector pORA-KUTAP were injected

intravenously into 10 mice per group (inoculum: 1 x 10^6 cfu) and followed until moribund. The survival times were not significantly different between wild-type *C. neoformans* (H99) and the two strains containing the KUTAP plasmid (mean time of death; H99 wt: 11 +/- 0.5 d, KUTAP 1: 12.6 +/- 0.5 d, KUTAP 2, 12.5 +/- 0.4 d, (SEM), p >0.05 by ANOVA between all groups). Thus, because the episome is able to confer wild type virulence through complementation of the uracil auxotrophy, we are able to utilize episomal expression to explore the cell biology of the pathogen within the model host.

4. Localization of laccase *in vitro* and *in vivo.*

4.1 Initial localization of laccase in cells grown *in vitro*.

The importance of laccase activity as a surface-active enzyme has prompted several investigations into its localization. Accurate localization of wild-type protein is a requirement for any initial characterization, as the use of tagged fusion proteins may result in mis-localization due to inteference with trafficking sequences [15, 16]. Early descriptions of localization were focused more on purification than localization and described an enzyme within the intracellular space as well as lipid membranes based on detergent studies [17]. Later studies using a combination of immuno-gold electron microscopy and a laccase monoclonal antibody demonstrated that laccase protein was predominately found within cell walls of fungal cells grown in vitro, with a minor portion secreted into the extracellular space during infection as well as in vitro [18, 19 and unpublished results]. Initial attempts at green fluorescent protein (GFP) localization using C-terminal tags resulted in some cell wall localization as well as intracellular targeting [19, 20] . Because of a lack of robust cell wall localization of the C-terminal tag, and poor intensity of fluorescence of ascomyete and mammalian GFP, newer basidiomycete-specific GFP proteins were developed and an N-terminal GFP fusion was selected for studies involving mammalian infections. Development of robustly expressed and localized GFP-tagged protein fusions can be useful for studying pathogenesis of fungi such as *C. neoformans* as the cells can be assessed for localization immediately after isolation from lung or brain or can be visulaized in real-time during macrophage infections. In adition, localization does not require fixation or sectioning that might perturb the native localization of the protein.

4.2 Construction of an N-Terminal Laccase-GFP Fusion

Because laccase is a secreted protein, it contains a leader peptide on the pro-enzyme that is cleaved during the maturation process. To ensure that the N-terminal GFP-laccase fusion protein would be properly processed and trafficked through the secretory pathway, the GFP was cloned in-frame between the leader peptide and the mature enzyme. The fusion was expressed from the native *LAC1* promoter in hopes of retaining not only localization during *in vivo* studies, but also mimicking the timing and magnitude of laccase expression during infection. The N-terminal GFP-laccase fusion was built in the episomal expression vector pORA-2XK (see above and Fig. 3) and retained under selective pressure of the *URA5* gene during infection. The vector was digested with I-*Sce1* to expose the telomeric ends, precipitated and used to transform a *ura*$^-$ derivative of the *lac1*Δ mutant. The *lac1*Δ mutant was chosen as the recipient strain so that any laccase activity would be due only to the GFP-tagged protein. Transformants were tested for the presence of the vector by assessing laccase activity and by epifluorescence microscopy. One transformant found to express wild type amounts of laccase activity was subjected to further analysis.

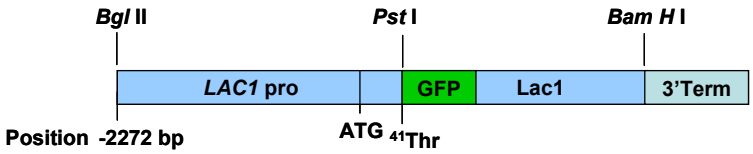

Fig. 3 N-terminal GFP-laccase fusion expression vector. Indicated construct containing the laccase promoter (*LAC1* pro), GFP, laccase coding region (Lac1) and a 3' terminator from EF1-alpha (3'Term) was subcloned into a pORA-2XK shuttle vector. Copyright © 2007, American Society for Microbiology.

4.3 Laccase localization *in vitro*

Under standard induction conditions (0% glucose, 30°C, pH 7.4), the *C. neoformans* strain expressing the N-terminal GFP-laccase fusion exhibited a strong ring of GFP fluorescence that co-localized with the cell wall (Fig. 4) which was visualized by staining with the chitin-binding dye calcofluor white. This confirmed the ability of the GFP-tagged construct to robustly traffick to the cell wall, similar to that exhibited by the wild-type enzyme. However, when the pH of the induction medium was reduced to pH 5.4, the GFP fluorescence was also found to be intracellular, associated with vesicles. These results suggested that the trafficking of laccase to the cell wall is sensitive to extracellular pH.

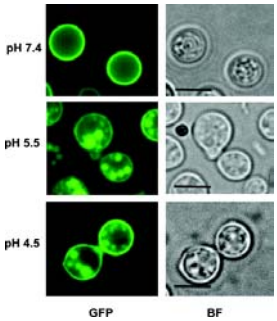

Fig. 4. Localization of GFP-laccase at neutral and acidic pH. *C. neoformans* cells expressing GFP-laccase were induced for laccase in asparagine media at the indicated pH for 24 h at 30°C and observed for epifluorescence. (1000x; bar indicates 5 μm). Copyright © 2007, American Society for Microbiology.

4.4 Laccase localization during mammalian infection.

C. neoformans has been demonstrated to be a facultative intracellular pathogen and alveolar macrophages have been found to be the first line of defense during lung infections [21]. Thus, to determine the localization of laccase during intracellular residence within lung macrophages, NIH Swiss ablino mice were inoculated intranasally with $5x10^4$ cells of a GFP-laccase expressing cryptococcal strain followed by mouse sacrifice and broncho-alveolar lavage (BAL). The BAL fluid was concentrated by centrifugation and GFP-laccase localization in yeast cells was assessed by deconvolution epifluorescent microscopy. As shown in Fig. 5, after infection by alveolar macrophages, GFP fluorescence was found to be associated with the cell wall with significant fluorescence seen in the cytoplasm. Macrophage autoflorescence observed in all sections is a predominant feature of macrophages and necessitates the inclusion of negative controls during infection fluorescent studies. The failure to achieve complete cell wall localization of the GFP-laccase construct suggests specific anti-trafficking mechanisms of macrophages that serve to combat anti-oxidant features of laccase in alveolar macrophages previously described [22].

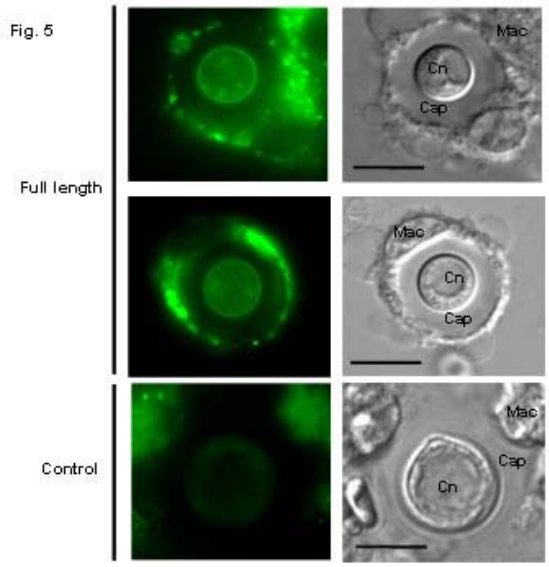

Fig. 5. Localization of laccase during infection of broncho-alveolar macrophages. Expression of GFP-laccase during infection of bronchial alveolar cells 48 h after inoculation. Control indicates infection with equivalent cells expressing an empty episomal vector (1000x; bar indicates 5 μm; Mac-macrophage, Cn-*C. neoformans* cell, Cap- fungal capsule). Copyright © 2007, American Society for Microbiology.

To investigate the production and localization of laccase during infection of the murine model host, a combination of both mouse models were employed. To evaluate laccase expression in the lung, CBA/Jcr and NIH Swiss albino mice were injected intranasally with $5x10^4$ cells of the GFP-laccase expressing strain. Laccase production and localization was visualized using deconvolution epifluorescence microscopy. As shown in Fig. 6, laccase was found in the cytoplasm in vesicles of both mouse strains with some accumulation in the cell wall of CBA/Jcr mice. In the brains of NIH Swiss albino mice injected intravenously with $1x10^6$ cells of the GFP-laccase expressing strain, GFP-laccase was almost exclusively found to be associated with the cell wall, facilitating its predominant role during brain infection (Fig. 6). These studies suggest that during adaptation to the host, *C. neoformans* displays laccase on the cell wall in response to specific environmental cues in the phagosome and within the CNS that may lead to neurotropism of this pathogen. Future studies will attempt to define cellular cues promoting cell surface association of laccase in these pathogenic niches.

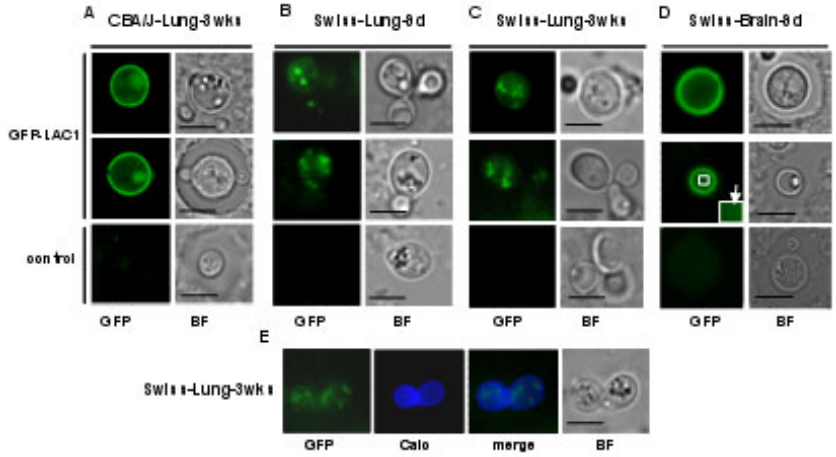

Fig. 6. Expression of GFP-laccase during infection of lung and brain. *C. neoformans* cells expressing GFP-LAC1 or a plasmid not containing GFP-LAC1 (control) were inoculated intranasally (10^5) into CBA/J mice (panel A) or Swiss-albino (panel B and C). Fungal cells recovered from homogenized lung and observed for epi-fluorescence. Similar cells were inoculated intravenously (10^6) into Swiss Albino mice and recovered from homogenized brain and observed for epi-fluorescence (panel D). Inset represents enlargement of indicated region; arrow shows cytoplasmic vesicle. (1000x; bar indicates 5 μm). Copyright © 2007, American Society for Microbiology.

5. Copper sensing, genetic diversity and neurotropism.

5.1 Copper acquisition during infections.

Metals such as copper and iron are essential to the function of all living cells. Both host cells and pathogens have devised strategies to control copper acquisition. For example, optimum copper homeostasis has been associated with the virulence of a number of intracellular pathogens including *Listeria monocytogenes* [23], *Mycobacterium tuberculosis* and *M. leprae* [24]. The presence of specific host mechanisms to deprive the pathogen of copper is suggested by the time-dependent reductions of copper within the phagolysosome reported to occur after uptake of intracellular pathogens such as *Mycobacteria*, as measured by a hard x-ray microprobe [25]. Copper is particularly important for melanization in *C. neoformans*, as laccase is a copper-dependent enzyme. Mutants of *C. neoformans* that fail to insert copper into the active site of laccase, such as a *vph1*Δ strain that is defective in vacuolar acidification, produce laccase protein that is appropriately targeted but is inactive [26]. In addition to laccase, many essential enzymatic functions rely on copper, such as the mitochondrial cytochrome complex (ref).

However, the important question related to pathogenesis is whether copper is limiting during mammalian infection. Thus, to study the role of copper in cryptococcal virulence we analyzed a copper-dependent mutant of the copper regulator, Cuf1 and found that copper dependence correlated with reduced virulence in a mouse model [27]. Furthermore, as shown in Fig. 7, we found that clinical isolates induced under identical conditions from 24 organ transplant patients expressed levels of a *C. neoformans* Cuf1-dependent high-affinity copper transporter, *CTR4,* that correlated with a propensity for brain dissemination vs. pulmonary disease alone ($p = 0.04$) [27]. Interestingly, a secondary analysis of patients controlling more rigorously for immunosuppression by excluding those receiving a less optimal non-anti-calcineurin-based regimen for their transplant (isolates 11, 12, 14, 17 and 18 below) actually increased the significance of the *CTR4*-brain dissemination relationship (p value < 0.01). This suggested a role of high affinity copper uptake mechanisms in the neurotropism of the fungus during human infections, requiring the development of cell biological tools to assess copper sensing by *C. neoformans* during infection.

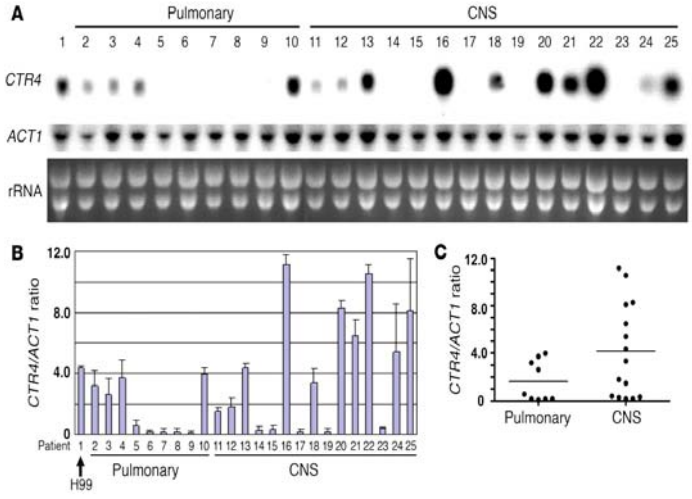

Fig. 7 Correlation of *CTR4* expression with CNS infection in clinical isolates of *C. neoformans.* A) 24 *C. neoformans* primary isolates were obtained from consecutive patients (or an H99 laboratory strain) as described in the text. Cells were induced by inoculation in asparagines salts and RNA obtained and subjected to northern blot, using a fragment of the indicated gene. rRNA was visualized by ethidium-bromide staining of gels. B) Northern blots were conducted in duplicate and *CTR4/ACT1* ratios calculated for the indicated strain by densitometry. C) Plot of average *CTR4/ACT1* values vs. organ involvement. Horizontal line indicates mean value for each group (p = 0.04). Copyright © 2007, American Society Clinical Investigation.

5.2 From the bedside to the molecule.

Because *CTR4* expression correlated strongly with extrapulmonary dissemination in clinical isolates from transplant patients, we set out to assess copper sensing *in vivo* through analysis of *CTR4* promoter activity [27]. The *C. neoformans cuf1*Δ mutant failed to induce expression of *CTR4,* encoding the high affinity copper transporter. Taken together, these results suggest that *CTR4* expression could be a marker of neurotropism in strains of *C. neoformans.*

5.3 Construction of the *CTR4* reporter strain.

To assess induction of the *CTR4* promoter within tissues of the model host, a reporter construct in which expression of GFP is driven by the *CTR4* promoter was designed. The construct was introduced into a *ura*$^{-}$ derivative of wild type *C. neoformans*. In order to test the induction of the *CTR4* reporter under copper starvation conditions the reporter strain was exposed to the copper chelator BCS. As predicted, the *CTR4* reporter was induced strongly in the presence of BCS and was strongly repressed in the presence of Cu^{2+}. Because the reporter construct exhibited regulation consistent with that of the wild-type gene, the reporter strain was used to assess copper sensing *in vivo.*

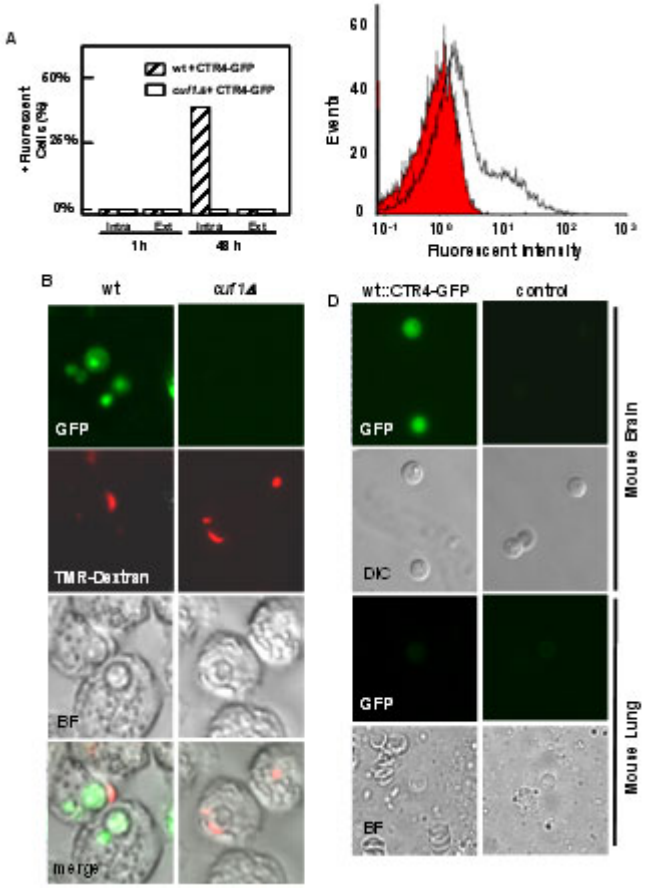

Fig. 8. Expression of *CTR4* in macrophages and mouse brain demonstrates low copper availability during infection. A) Wild-type (wt) or *cuf1Δ* cells expressing equivalent CTR4-GFP constructs under the *CTR4* promoter were incubated with the macrophage-like cell line, J774.1 for indicated times; 200 intracellular (intra) and extracellular (ext) were scored for the presence of epifluorescence at the indicated time points. B) Representative intracellular fungal cells from A, visualized by epifluorescence and intracellular co-localization by TMR-labelled dextran after phagocytosis as described in Materials and Methods. (1000x) C) *C. neoformans* cells expressing either CTR4-GFP or empty plasmid alone was injected (10^6 cells) by tail vein into Swiss Albino mice and after sacrifice, fungal cells were harvested from brain and epifluorescence measured by flow cytometry. Unshaded curve corresponds to signal from CTR4/GFP expressing cells, shaded to signal from cells expressing an equivalent construct without CTR4/GFP. D) Representative cryptococcal cells from C, visualized by microscopy after recovery from brain or after recovery from lung of intranasally injected CBA/J mice (1000x).

5.4 *In vivo* analysis of *CTR4* induction

Because macrophages have been demonstrated to sequester nutrients from invading organisms, the induction of *CTR4* was assessed during growth in the J774.16 macrophage-like cell line. As shown in

Fig. 8, *CTR4* promoter activity was strongly induced inside the phagosome of macrophages suggesting that copper sequestration could be an important anti-pathogenic mechanism of macrophages. To test potential roles of copper acquisition during whole animal infection, the *CTR4* reporter strain was injected into the tail vein ($1x10^6$ cells) of NIH Swiss Albino mice or intranasally inoculated ($5x10^4$ cells) into the lungs of CBA/Jcr mice. After harvest from brain or lung, cells were analyzed for GFP fluorescence using deconvolution epifluorescence microscopy. Interestingly, no *CTR4* reporter expression (GFP fluorescence) was detected in yeast cells taken from the lungs. However, GFP fluorescence was robust in cells recovered from the brains of mice, indicating strong induction of the *CTR4* promoter (Fig. 8). Since induction of the *CTR4* promoter is stimulated by copper depletion [27], the *in vivo* induction of the *CTR4* promoter suggests that the CNS is an environment in which copper is limiting to *C. neoformans,* requiring the induction of the high-affinity copper transport system. This finding is consistent with *CTR4* expression being indicative of a pathogen with increased disease severity during an extrapulmonary brain infection and further implicates a role for copper sequestration in anti-pathogen mechanisms by the mammanlian host.

6. Endless Possibilities

The previous sections illustrate two scenarios in which cell biological methods have been applied to a pathogen within the context of the host environment. Using protein localization, we were able to illustrate differences in targeting of laccase within different tissues during infection, illustrating a potentially novel property of macrophages- inhibition of virulence factor trafficking. Using reporter analysis, we were able to demonstrate that the CNS and macrophage phagosomes represent environments where *C. neoformans* have limited access to copper. These studies also revealed a potential clinical relationship between increased *CTR4* expression and CNS dissemination on organ transplant patients. These represent the simplest application of cell biology in the context of the host, but only a beginning. As new questions of pathogenesis arise, it will necessitate the design of compatible molecular tools to provide relevant answers in the context of the mammalian host. Avenues for exploration include the use of RNA interference to analyze the function of essential genes in the host, use of tissue-specific promoters to create programmatic gene silencing in *C. neoformans* infection and generation of additional *C. neoformans* codon-optimised fluorescent proteins to be used for *in vivo* colocalization experiments. Through these and other approaches we can go beyond assessment of virulence as a simple presence or absence of a gene or "virulence factor", and probe the intricacies of the dynamic cellular processes that determine and modulate pathogenesis within the host.

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