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ORIGINAL PAPER

Effects of *CTR4* deletion on virulence and stress response in *Cryptococcus neoformans*

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Abstract Roles of the high-affinity copper transporter Ctr4 in the virulence of *Cryptococcus neoformans* remain to be fully determined. Here we demonstrate that Ctr4 plays a necessary role in virulence and tolerance to a number of stress conditions. We first observed, with the method of flame atomic absorption spectrometry, that deletion of *CTR4* resulted in a significant decrease in intracellular copper level, confirming the role of Ctr4 as a copper transporter in *C. neoformans*. Furthermore, *CTR4* was critical for the yeast to survive at both elevated and low temperatures, as the growth rate of the *ctr4Δ* mutant at 4 and 37 °C was significantly decreased. The mutant *ctr4Δ* also exhibited hypersensitivity to osmotic stress imposed by 2 M NaCl or KCl, indicating the possible crosstalk of Ctr4 with the HOG signalling pathway. Moreover, cell wall and plasma membrane integrity appeared to be impaired in the *ctr4Δ* strain. The virulence of *ctr4Δ* in two mouse cryptococcosis models was remarkably reduced either via an intranasal or intravenous inoculation. Our work confirms the roles of Ctr4 in virulence and copper homeostasis as well as other additional novel functions.

Keywords Copper uptake · *Cryptococcus neoformans* · *CTR4* · Osmolality · Virulence

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Introduction

The basidiomycetous yeast *Cryptococcus neoformans* is an important fungal pathogen clinically and causes systemic infections in immunocompromised hosts such as patients with AIDS, resulting in approximate 600,000 deaths annually (Park et al. 2009). As a model system for the study of fungal pathogenesis, several virulence-associated traits have clearly been identified, including the formation of polysaccharide capsule, the production of antioxidant melanin and the ability to grow at 37 °C (Zhu and Williamson 2003; Janbon 2004; Zaragoza et al. 2009).

Several reports have described the importance of copper (Cu) homeostasis in virulence of *C. neoformans*. As an essential trace nutrient, copper can easily transit between two oxidation states, Cu^{+} and Cu^{2+} (Van Ho et al. 2002), providing a suitable chemical environment for various biological processes. Production of the pigment melanin depends on the copper-

containing laccase. Endogenous copper induces laccase expression and melanin production in *C. neoformans* (Zhu and Williamson 2004; Jiang et al. 2009). Disruption of the chloride channel (*CLC*) also reduces laccase activity, whereas exogenous copper supplementation restores the phenotype (Jiang et al. 2009). Moreover, a mutant of the secretory compartment copper pump (*CCC2*) also showed reduced virulence and copper deficiency phenotypes (Walton et al. 2005). A copper-dependent transcription factor Cuf1 has been defined to be required for copper induction of laccase in *C. neoformans* (Jiang et al. 2011). Deletion of *CUF1* leads to defects in growth and reduced expression of virulence factors in low copper conditions (Waterman et al. 2007). Cuf1 plays dual roles in both copper detoxification in response to Cu excess and high-affinity uptake under Cu limitation (Ding et al. 2011; Jiang et al. 2011). High-affinity copper uptake is mediated by two plasma membrane transporters encoded by *CTR1* and *CTR4* in this yeast (Sun et al. 2014). Expression of cryptococcal *CTR1* or *CTR4* both rescues the cell growth defect of a *S. cerevisiae ctr1Δ/ctr3Δ* strain, indicating the independent role of these two copper transporters (Ding et al. 2011; Sun et al. 2014).

As a participant in the sophisticated mechanisms of copper homeostasis, Ctr4 was the first high-affinity copper transporter identified in *C. neoformans* (Ory et al. 2004). Surprisingly, conflicting observations have been reported regarding its role in the virulence of *C. neoformans* (Waterman et al. 2012; Sun et al. 2014). Thus, we decided to make a deletion mutant of *CTR4*, attempting to clarify this issue and to gain a better understanding of Ctr4-mediated physiological processes as a copper transporter. By flame atomic absorption spectrometry, we first confirmed its crucial function in copper uptake. We found several undescribed phenotypic outcomes of *CTR4* deletion in extreme temperature tolerance and osmotic resistance. In particular, we observed a decreased virulence for the mutant in two different mouse cryptococcosis models.

Materials and methods

Strains and culture conditions

C. neoformans var. *grubii* H99 is the wild-type strain. H99FOA, a uracil auxotrophic mutant derived from H99, was employed as the recipient strain for target gene deletion (Toffaletti et al. 1993; Zhu and Williamson 2004; Jiang et al. 2009). Yeast strains were routinely cultured in YPD medium (2 % glucose, 1 % yeast extract, 2 % Bactopeptone, and pH 6.0). Asparagine salt medium (0.1 % glucose, 0.1 % asparagine, 0.3 % KH_2PO_4, pH 5.2, Asn) containing 100 mg/l nor-epinephrine (NE) and 0.05 % $MgSO_4$-$7H_2O$ was used to test the laccase-mediated melanin production. Synthetic minimal medium, YNB (0.17 % amino acid-free yeast nitrogen, 0.5 % ammonium sulphate, and 2 % glucose) was employed for growth test. Strains were incubated at 28 °C unless indicated.

Disruption and complementation of *CTR4*

To generate the targeted null mutant for *CTR4* (CNAG_00979), a 2.3-kb genomic fragment containing the whole length of *CTR4* gene was PCR amplified and cloned into the vector pBluescript SK (+). Then, a 1.9-kb PCR-amplified fragment of the *URA5* gene was inserted into the *CTR4* fragment at the S*ac*II site. *C. neoformans* strain H99FOA was then transformed by electroporation (Jiang et al. 2009). Candidate transformants were primarily selected on YNB medium and further screened by PCR amplification. The deletion candidates were confirmed by Southern blot analysis. To reconstitute the wild-type allele, a 2.0-kb *CTR4* fragment was amplified from the genome of H99 using primers CTR4-C1/CTR4-C2 (5′-CCGTCTAGAGATGTCCCATTGCGCTGATA/5′-GTACTCGAGGCGGACGCATTCAAGGTTCA) and inserted into the plasmid pBS-hyg, which contained a hygromycin resistance cassette for selection. The complemented strain was also confirmed by PCR amplification and Southern hybridization.

DNA preparation and Southern blotting

Genomic DNA was prepared as described before (Li et al. 2012). Digestion of wild-type genome with S*al*I would produce a 2.9-kb signal band, when hybridizing with the *CTR4* probe. In contrast, the genome of the mutant treated in the same way would generate two signal bands (2.4 and 1.1 kb) (Fig. 1) because of the existence of two S*al*I restriction sites within the *URA5* marker. Apart from the two bands identified in the *ctr4Δ* mutant, the reconstituted strain would produce a third band resulting from gene complementation

(Fig. 1). The same fragment of *CTR4* used on the complemented cassette was applied as probe (2.0 kb) and labelled with [α-^{32}P]-dCTP using the Random Primer DNA Labelling Kit (TaKaRa Biotech Co. Ltd. Dalian, China). Digested DNA was subject to 0.8 % agarose gel separation and transferred to XH395 Magaprobe Nylon Transfer Membrane (GE OsmonicsInc, MN, USA).

Quantification of intracellular copper by flame atomic absorption spectrometry

To determine the quantity of intracellular copper, flame atomic absorption spectrometry (FAAS) was employed as described previously (Rae et al. 1999; Jiang et al. 2011). Briefly, yeast cells were collected and inoculated to 25 ml Asn liquid medium (2 % glucose) supplemented with 0, 5 or 50 μM $CuSO_4$. The concentration of each culture was diluted to OD_{600} of 0.1. After shaking at 28 °C for 45 min, 2×10^8 cells were taken out and washed three times with sterile ddH_2O, which had been treated beforehand with Chelex-100 (Bio-Rad Laboratories Inc., Hercules, CA, USA). Cells were lyophilized and resuspended in 2 ml nitric acid, and then the suspension was heated at 85 °C till the solution became clear. The quantity of copper was determined by Hitachi 180-80 AAS machine according to the manufacturer's instructions (Hitachi Ltd, Japan). Wild-type H99 and the complement were used as controls in parallel. The assay was performed in triplicate, and errors were expressed as the standard deviation.

Virulence test in mouse cryptococcosis models

Virulence studies were conducted using a previously described mouse meningoencephalitis model (Yang et al. 2010). Yeast cells were incubated overnight in liquid YPD medium, washed and suspended in physiological saline. Cell numbers were estimated by haemocytometer and confirmed by colony formation on YPD agars. For the intravenous model, 6–10-week-old BALB/c female mice were anaesthetized and inoculated with 1×10^4 cells of each yeast suspensions in 0.1 ml via the lateral tail vein. For the pulmonary model, C57BL/6 mice were anaesthetized and inoculated intranasally with 3×10^7 cells in 50 μl physiological saline. Groups of ten mice were monitored daily for survival. Mice unable to reach food or water were sacrificed and counted as dying that day. For fungal burden assays, organs (lung and brain) were removed under sterile conditions from sacrificed mice, weighed and homogenized in sterile PBS buffer. Serial dilutions of the suspensions were plated on YPD agars at 28 °C for 3 days, and colonies were counted after incubation.

Results

Targeted disruption of *CTR4* gene

To further characterize the role of *CTR4* in *C. neoformans*, we made a targeted deletion of the gene. The ORF of *CTR4* was disrupted by *URA5* via

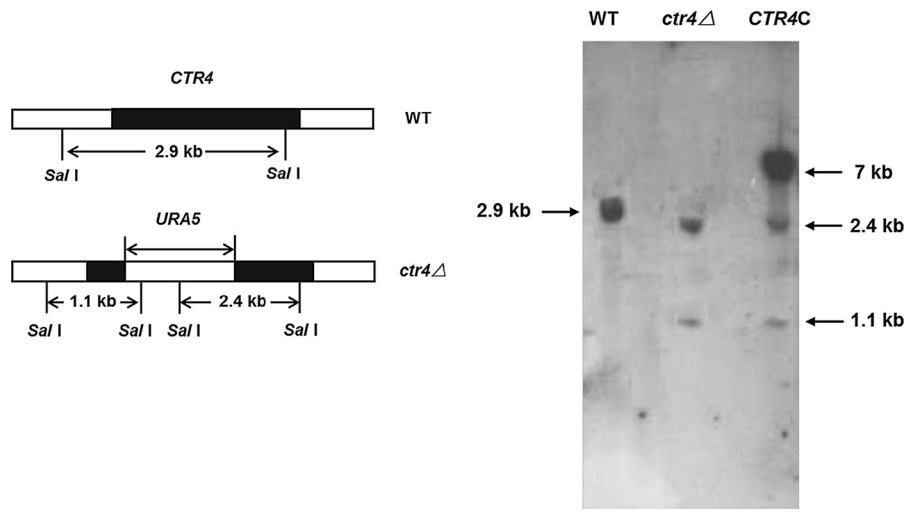

Fig. 1 Disruption and Southern blot analysis of the *ctr4Δ* mutant. The ORF of *CTR4* was replaced by *URA5*. Genomic DNA from wild-type H99, *ctr4Δ* and the complement was digested with S*al*I, and probed with a 2.0-kb *CTR4* fragment. Digestion of the wild-type genome yielded a 2.9-kb band, whereas the mutant had two bands, 1.1 and 2.4 kb in length. The reconstituted strain exhibited the third band resulted from the gene complement

homologous recombination as demonstrated by Southern blotting (Fig. 1). By screening with PCR amplification, the correct mutant, *ctr4Δ*, was obtained. Mutant strain *ctr4Δ* was then complemented with a 2.0-kb genomic fragment of the wild-type *CTR4* gene and a hygromycin resistance cassette. The correctness of the *ctr4Δ* mutant and the complemented strain *CTR4*C was verified by Southern blotting (Fig. 1). The reconstituted strain restored the expression of *CRT4* (data not shown).

Intracellular copper deficiency in *ctr4Δ* mutant

To ascertain the effect of *CTR4* deletion on copper uptake, we tested the quantity of intracellular copper in the *ctr4Δ* mutant by FAAS. Wild-type H99 and the complement served as control. The intracellular levels of copper in *ctr4Δ* fell significantly to 0.66 ± 0.02 μg in 2×10^8 cells, while the value of H99 and the complemented strain were 2.01 ± 0.04 and 1.41 ± 0.03 μg respectively (Fig. 2). Thus, disruption of *CTR4* leads to a defect in copper uptake in the *ctr4Δ* mutant, suggesting a role for Ctr4 as a copper transporter in *C. neoformans*.

As one of the crucial trace nutrients, we wondered whether the decreased copper content had marked effects on the activities of copper-containing elements in *ctr4Δ*. Thus, two of the essential indexes, Cu/Zn-SOD activity and cytochrome *c* oxidase activity were assayed to test the copper-requiring processes. On

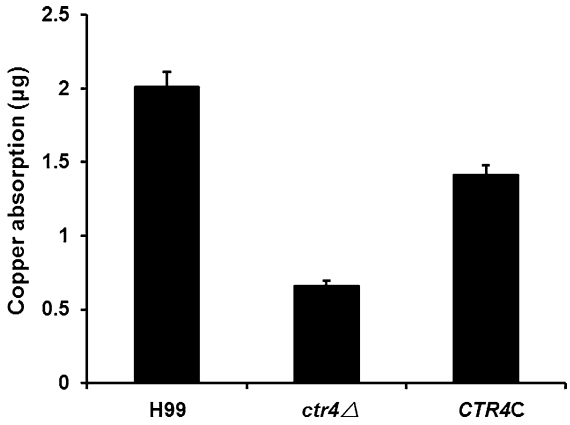

Fig. 2 Intracellular copper deficiency in *ctr4Δ*. The copper quantity in 2×10^8 cells collected from copper starvation conditions was determined by FAAS. The intracellular copper in *ctr4Δ* was 0.66 ± 0.02 μg per 2×10^8 cells, whereas the amount for H99 and the complemented strain was 2.01 ± 0.03 and 1.41 ± 0.06 μg, respectively

YPD, the *ctr4Δ* mutant exhibited smaller colonies compared with the wild-type H99 (Fig. 3a). However, on 3 μg/ml of menadione, a superoxide generator that affects Cu/Zn-SOD activity (Cox et al. 2003), the *ctr4Δ* mutant showed a discernible growth delay (Fig. 3b) taking into account of its retarded growth on YPD (Fig. 3a). Similarly, respiratory carbon sources, as the index for cytochrome *c* oxidase activity, had modest effects on growth rates of *ctr4Δ* (Fig. 3c), consistent with the decreased intracellular level of copper. This result is consistent with a previous report (Ding et al. 2011).

Critical roles of *CTR4* in extreme temperatures

We found that *ctr4Δ* is sensitive to elevated temperature, as the growth rate of *ctr4Δ* at 37 °C on YPD agar was investigated. Deletion of *CTR4* resulted in slight delays in growth as compared with H99 and the complemented strain under 28 °C condition (Fig. 4a). When the mutant was grown at 37 °C, the defect became severer (Fig. 4a). Reconstitution of Ctr4 restored the growth at high temperature. Interestingly, the *ctr4Δ* mutant exhibited severe growth defects at low temperatures, such as 4 °C (Fig. 4a). Exogenous copper could not restore the temperature tolerance defect (Fig. 4b).

To further confirm the growth defect of the *ctr4Δ* mutant, we constructed a growth curve under various test conditions. As shown in Fig. 6a, it was apparent that the *ctr4Δ* mutant exhibited a decreased growth rate at 37 °C. The result was consistent with the results on YPD agars. Unfortunately, due to the severe growth defect for the mutant at 4 °C, we were unable to obtain a growth curve assay in this condition. These results imply a relationship between copper uptake and temperature adaptability.

Critical roles of *CTR4* in osmolality stress tolerance

Further findings include the tolerance roles of *CTR4* to other stresses. We found that, compared with H99 and the complemented strain, the mutant displayed a high sensitivity to 2 M KCl or NaCl. The reconstituted cells restored growth on plates with high concentrations of salt (Fig. 5a), which suggested a critical role of *CTR4* in response to osmolality stress. Again, to ensure that the *ctr4Δ* mutant failed to resist the stresses imposed

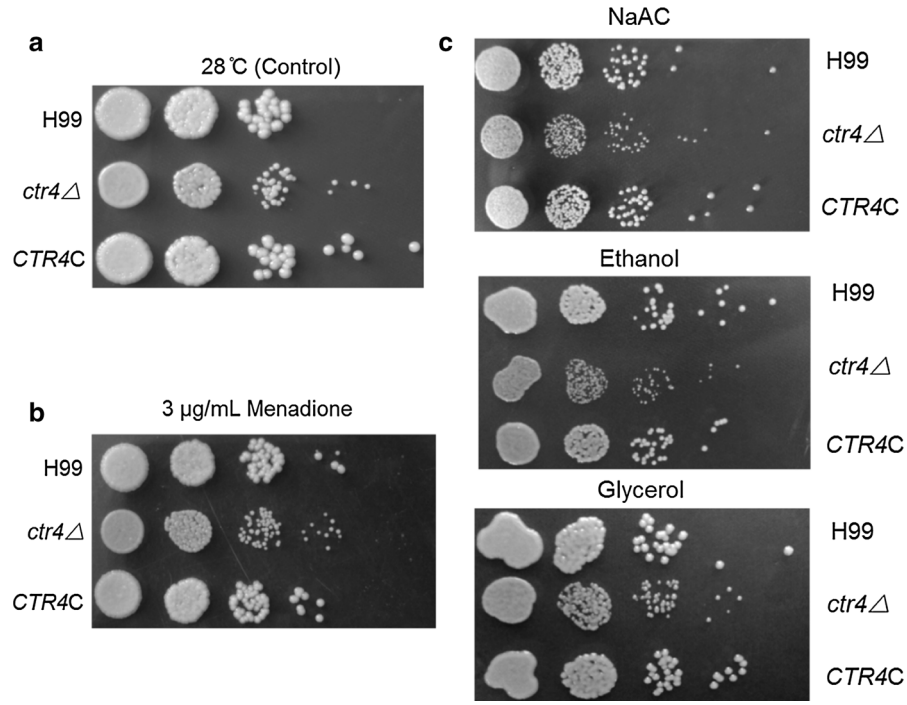

Fig. 3 Effects of *CTR4* deletion on phenotype. Starting with 2×10^6 cells/ml, yeast cells were diluted with tenfold serial dilution and 3 μl of the diluted suspension spotted on agars as indicated at 28 °C for 3 days. H99 and the complement served as control. **a** Growth rate of *ctr4Δ* mutant on YPD agar at 28 °C. **b** Sensitivity of *ctr4Δ* to 3 μg/ml menadione. **c** Growth rate of *ctr4Δ* mutant on non-fermentable carbon sources

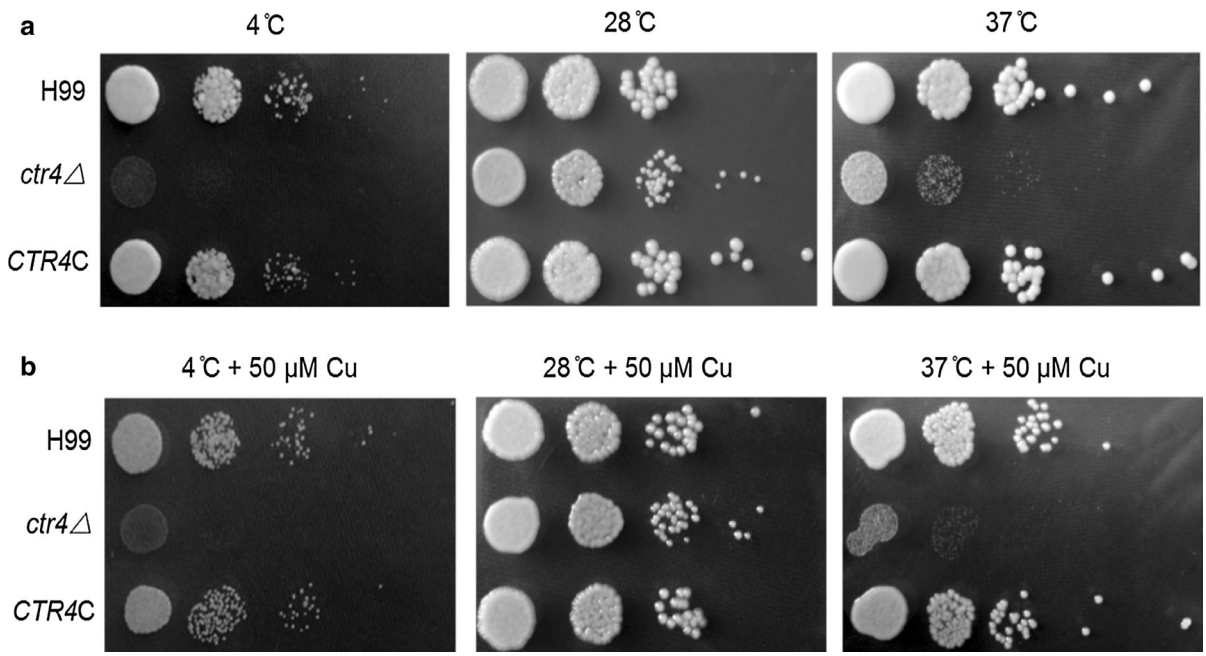

Fig. 4 Requirement of *CTR4* for thermotolerance. Starting with 2×10^6 cells/ml, 3 μl of the serially diluted suspension was dropped on YPD agars for 3 days as indicated. H99 and the complement served as control. **a** Critical roles of *CTR4* in temperature adaptability. **b** Exogenous copper could not restore the temperature tolerance

by 2 M KCl and NaCl, we made growth curves under high osmotic pressure and observed a significant delay in the growth rate of *ctr4Δ* (Fig. 6b). We also noticed that high concentrations of salt in the medium apparently increased the lag phase, for both the wild-type H99 and *ctr4Δ* mutant. The above results indicate that *C. neoformans CTR4* plays a vital role in response to osmolality stress.

We also examined the resistance of *ctr4Δ* to oxidative stresses created by H_2O_2 and $NaNO_2$, and found that sensitivity of *ctr4Δ* to reactive oxygen and nitrogen species was similar to that of wild type (Fig. 5b) if taking into consideration of its retarded growth on YPD (Fig. 3a). However, the *CTR4* deficient strain exhibited obvious sensitivity to 0.01 % SDS and 1 % Congo red (Fig. 5c), suggesting defects in the cell membrane and cell wall integrity of the *ctr4Δ* mutant. Moreover, we confirmed a severe growth defect of *ctr4Δ* under nutrient limitation conditions with YNB minimal medium (data not shown), which was also observed by Waterman et al. (2012).

Effect of *CTR4* deletion on survival and fungal burden in mouse cryptococcosis models

The role of *CTR4* in the virulence of *C. neoformans* remains puzzling, as inconsistent results have been obtained by different studies (Waterman et al. 2012; Sun et al. 2014). Thus, we carried out virulence testing for the H99, *ctr4Δ* and the complemented strains in mouse cryptococcosis models. Initially, we used the intravenous model to test the virulence of the indicated strains though tail inoculations. As shown in Fig. 7a,

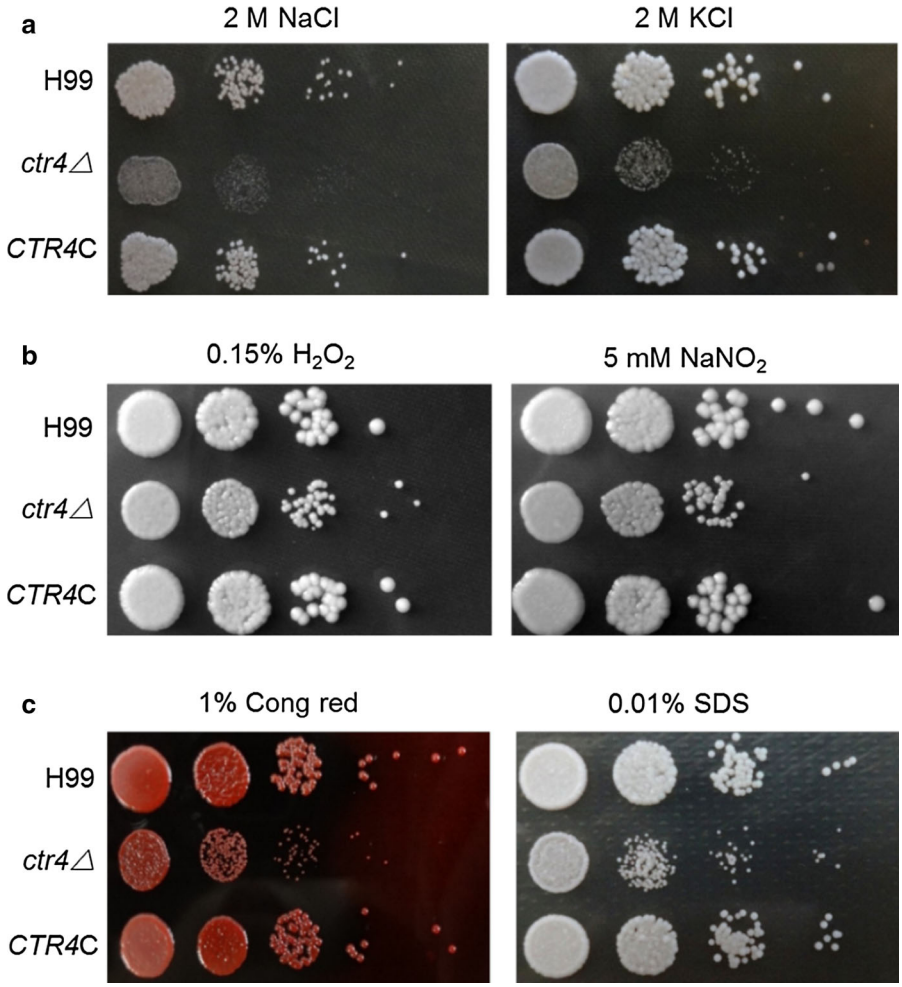

Fig. 5 Roles of *CTR4* in stress resistance. Starting with 2×10^6 cells/ml, 3 µl of the serially diluted suspension (1:10) was spotted on agars supplemented with testing agents at 28 °C for 3 days. The mutant strain *ctr4Δ* was highly sensitive to 2 M NaCl and KCl (**a**) but not to H_2O_2 (0.15 %) and $NaNO_2$ (5 mM) (**b**). *ctr4Δ* mutant exhibited mild sensitivity to Cong red (1 %) and SDS (0.01 %) (**c**)

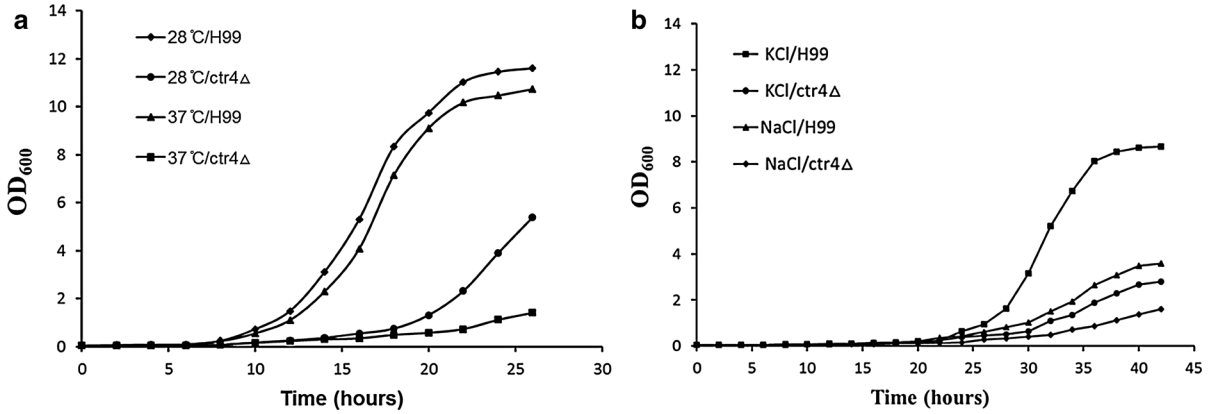

Fig. 6 Defects of *ctr4Δ* mutant in resistance to temperature and osmolality stresses by growth curve experiments. Overnight cultures were diluted with YPD liquid medium to an OD_{600} of 0.05, and the optical density was measured every 2 h until the growth curve of wild-type H99 entered into stationary phase. The results shown were the averages of three measurements. **a** Growth curves of *ctr4Δ* mutant in 28 °C and 37 °C conditions. The growth curves from top to bottom represent H99 in 28 °C, H99 in 37 °C, *ctr4Δ* in 28 °C and *ctr4Δ* in 37 °C, respectively. **b** Growth curves of *ctr4Δ* mutant in mediums containing 2 M NaCl and 2 M KCl. The growth curves from top to bottom represent H99 in 2 M KCl, H99 in 2 M NaCl, *ctr4Δ* in 2 M KCl and *ctr4Δ* in 2 M NaCl, respectively

infections with H99 and the complemented strain resulted in complete mortality after 20th and 22nd day post-infection respectively. However, the *ctr4Δ* strain-infected mouse survived for more than 40 days after infection ($P < 0.01$), exhibiting apparent attenuated virulence compared with the wild type and reconstituted strains.

Subsequently, we conducted virulence testing using a pulmonary model. Data of mortality exhibited a parallel result of attenuated virulence resulting from deletion of *CTR4* as in the intravenous model (Fig. 7b). In the pulmonary model, the *ctr4Δ* strain-infected mouse survived to the 29th date after infection ($P < 0.01$), while infections with H99 and the complement resulted in complete mortality after the 19th and 24th day post-infection, respectively. An additional fungal burden analysis of lungs and brains was performed in the pulmonary model. Interestingly, we detected striking increases in fungal burden in the lungs of mice infected with *ctr4Δ* mutant (Fig. 7c), suggesting that deletion of *CTR4* favours the yeast to survive in the high-copper lung. Notably the fungal burden in the brains decreased significantly (Fig. 7d). Cumulatively, environmental availability of copper (host organs) appears to play a critical role for the pathogen to colonize and thus virulence of the pathogen.

Discussion

The trace element copper has been demonstrated to play critical roles in the virulence of *C. neoformans* (Waterman et al. 2007; Jiang et al. 2009; Ding et al. 2011). Most of the knowledge about copper acquisition and storage comes from studies on non-pathogenic fungi (Peña et al. 2000; Ioannoni et al. 2010). Copper homeostasis as a virulence factor in *C. neoformans* has also been studied recently. Unfortunately, the role of the copper-affinity transporter *CTR4* in the pathogenesis remains contradictory based on previous reports (Waterman et al. 2012; Sun et al. 2014). In this report, we confirmed that the high-affinity copper transporter Ctr4 in *C. neoformans* is required for virulence in cryptococcosis mouse models, and additionally for copper acquisition, temperature adaptability and osmotic stress resistance. Several reports demonstrate that expression of *CTR4* is copper-dependent whose transcription is activated by copper deficiency and inhibited under copper excess, which was also observed by us (data not shown). In this paper, we provide FAAS data to show that disruption of *CTR4* resulted in marked reductions in intracellular copper level, suggesting its role in copper uptake (Fig. 2). Contrary to our expectations, the impaired copper absorption did not completely abolish

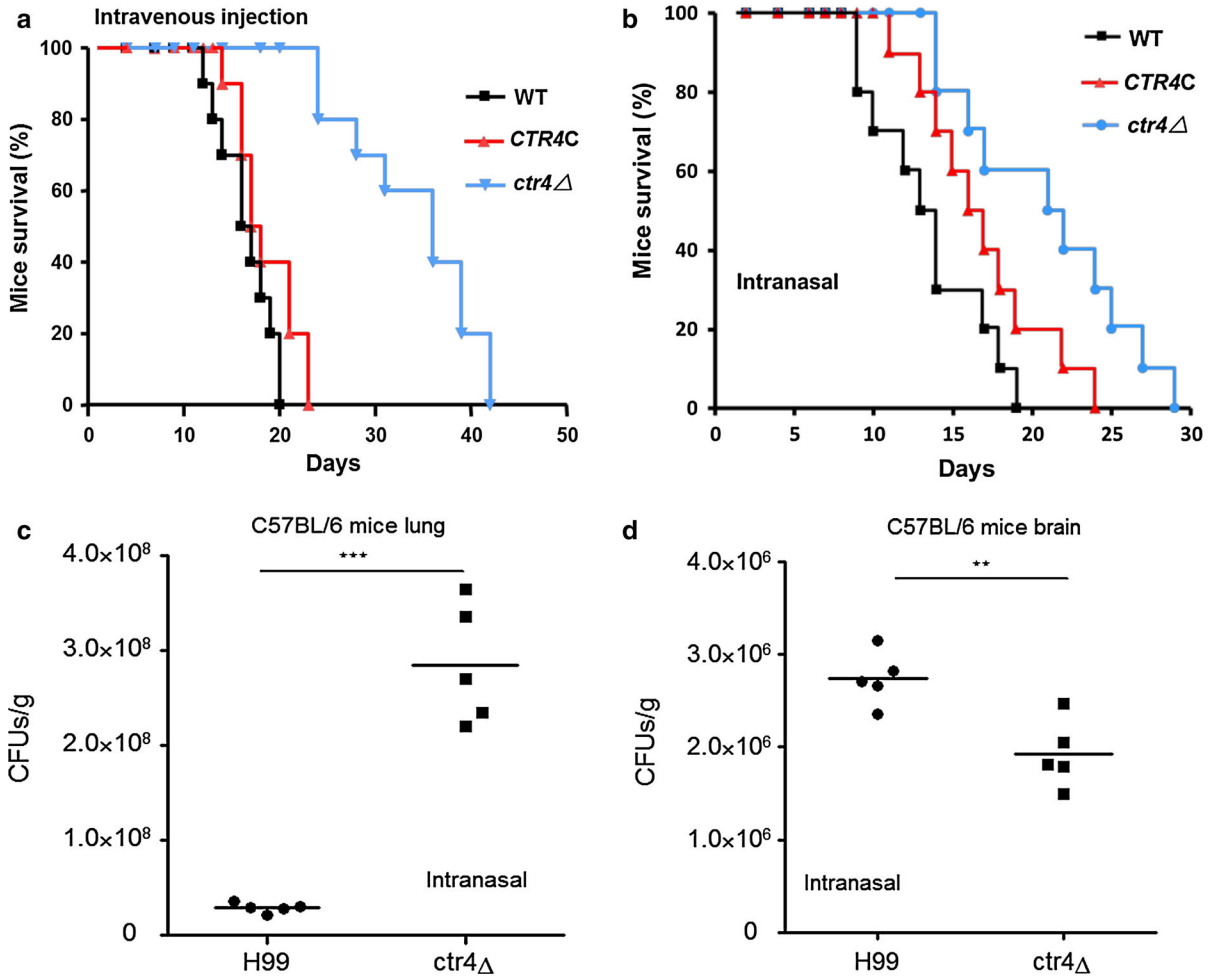

Fig. 7 The attenuated virulence of *ctr4Δ* in mouse cryptococcosis models. Mice were inoculated with *C. neoformans* intravenously and intranasally. Survival time and fungal burden were analysed. **a** BALB/c female mice were infected with H99, *ctr4Δ* and *CTR4*C cells via the lateral tail vein. **b** C57BL/6 female mice were infected intranasally with H99, *ctr4Δ* and *CTR4*C cells. **c**, **d** Fungal burden of the infected C57BL/6 mice in the lung and brain. CFUs were normalized to tissue weight. Significant differences are shown as indicated (***$P < 0.001$, **$P < 0.05$)

activities of copper-containing elements, including the laccase, Cu/Zn-SOD and cytochrome *c* oxidase. Taking into account the fact that *S. cerevisiae ctr1Δ/ctr3Δ* mutants are unable to grow in media containing non-fermentable carbon sources (Puig and Thiele 2002), we believe that other pathways such as *CTR1* may play overlapping functions in copper uptake (Sun et al. 2014).

As to the roles of *CTR4* in virulence, discrepancies in observation have been reported by two groups (Waterman et al. 2012; Sun et al. 2014). We demonstrated a remarkable attenuated virulence in this study for the mutant strain *ctr4Δ* in both pulmonary and intravenous models (Fig. 7). The consistent results we obtained with two infection models were in a general agreement with the observation by Waterman et al. (2012), however, in a striking discordance with Sun's result (2014) which presented a hypervirulent phenotype for their *ctr4Δ*. We noticed that the means used in construction of *ctr4Δ* by the groups were distinct. In our study and the Waterman report, *CTR4* was disrupted with the native cryptococcal *URA5* gene as selection marker, although Waterman's *ctr4Δ* contained a 255-bp deletion in the *CTR4* ORF, while our mutant resulted from an insertion of the *URA5* marker at the *Sac*II site. Sun's group created their mutant with

the drug resistant gene NEO which encodes a phosphorylase. We speculate that the selection marker NEO gene may boost the virulence for *C. neoformans* in the background of *CTR4* deletion. Unfortunately, this issue was not addressed by the authors but warrants further investigation.

The decreased virulence of the *ctr4Δ* mutant can be explained by the involvement of *CTR4* in tolerance for the yeast to various stress conditions. An interesting finding of this work is the role of *CTR4* in response to temperature variation (Fig. 4). Deletion of *CTR4* resulted in a hypersensitivity to low temperature 4 °C (Fig. 4). Unfortunately, little attention has been paid to the growth of this yeast under low temperature conditions. We also found retarded growth of the mutant at elevated temperature 37 °C (Fig. 4). We noticed that a different result as to the role of *CTR4* in elevated temperature tolerance was reported by Waterman et al. (2012) based on a spot plate assay. Thus, we further conducted the growth curve experiments and confirmed the growth defects of *ctr4Δ* mutant at high temperature (37 °C, Fig. 6). This difference may result from the inoculum and dilution factors they used which differed from ours. As the authors (Sun et al.) did not carry out a quantification test with growth curves, it is not easy to determine the exact cause for the discrepancy. In H99, a variety of elements have been shown to play critical roles in high-temperature tolerance, such as the Ras–Cdc24 signal pathway, the HOG pathway and a vacuolar H^+-ATPase gene *VPH1* (Erickson et al. 2001; Bahn et al. 2005; Nichols et al. 2007). Importantly, cell wall integrity is critical for heat stress resistance (Kraus et al. 2003). Since deletion of *CTR4* increased the sensitivity of yeast to 1 % Congo red and 0.01 % SDS (Fig. 5c), we believe that defects in cell wall/membrane integrity in our mutant is perhaps responsible for elevated-temperature sensitivity. Moreover, novel roles of *CTR4* in resistance to 2 M NaCl and KCl have been characterized (Fig. 5a). As it has been established that the high osmolality glycerol (HOG) pathway controls the resistance to osmotic stress in *C. neoformans* (Bahn et al. 2005), it seems that the Ctr4 transporter may interact with the HOG pathway. Taking together, the results indicate that as a membrane protein, Ctr4 may play various roles in addition to copper transporter in *C. neoformans*, such as signal transduction and maintenance of cell wall/membrane integrity. Our work, along with previous reports, provides further insight into the physiological roles of *CTR4* in *C. neoformans*.

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Compliance with ethical standards

Ethical approval All mice were housed and bred in a specific pathogen-free facility at Nankai University. All animal experiments were approved by the Animal Care and Use Committee at Nankai University.

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