The C2 Domain Protein Cts1 Functions in the Calcineurin Signaling Circuit during High-Temperature Stress Responses in

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Calcineurin is a conserved calcium/calmodulin-dependent serine/threonine-specific protein phosphatase that acts in cell stress responses. Calcineurin is essential for growth at 37°C and for virulence of the human fungal pathogen *Cryptococcus neoformans*, but its substrates remain unknown. The C2 domain-containing, phospholipid-binding protein Cts1 was previously identified as a multicopy suppressor of a calcineurin mutation in *C. neoformans*. Here we further characterize the function of Cts1 and the links between Cts1 and calcineurin. GFP-Cts1 localizes to cytoplasmic puncta and colocalizes with the endosomal marker FM4-64. The *cts1Δ* mutant shows a distinct FM4-64 staining pattern, suggesting involvement of Cts1 in endocytic trafficking. In large budded cells, GFP-Cts1 localizes transiently at the mother bud neck, as a single ring that undergoes constriction. mCherry-Cts1 colocalizes with the GFP-tagged calcineurin catalytic subunit Cna1 at sites of mRNA processing at 37°C, suggesting that Cts1 and calcineurin function coordinately during thermal stress. GFP-Cts1 exhibits slower electrophoretic mobility for cells grown at 37°C than for cells grown at 24°C, and the shift to a higher molecular weight is more pronounced in the presence of the calcineurin inhibitor FK506. In vitro treatment with calf intestinal alkaline phosphatase (CIP) restores faster electrophoretic mobility to GFP-Cts1, suggesting that Cts1 is phosphorylated at 37°C and may be dephosphorylated in a calcineurin-dependent manner. mCherry-Cts1 also coimmunoprecipitates with GFP-Cna1, with greater complex formation at 37°C than at 24°C. Taken together, these findings support potential roles for Cts1 in endocytic trafficking, mRNA processing, and cytokinesis and suggest that Cts1 is a substrate of calcineurin during high-temperature stress responses.

*Cryptococcus neoformans* is a human fungal pathogen that causes life-threatening meningitis, primarily in immunocompromised patients (5, 17). Cryptococcal meningitis is one of the most important HIV-related opportunistic infections, with ~1 million cases occurring each year (36). Identification of novel therapeutic targets for cryptococcosis is essential given the toxic effects of existing therapies and the rising prevalence of drug-resistant strains (38).

The ability to survive, grow, and divide at human physiological temperature is an important *C. neoformans* virulence attribute and is orchestrated by elaborate signaling pathways that are not yet fully elucidated (3, 19). Calcineurin, a calcium/calmodulin-dependent serine/threonine-specific protein phosphatase, is essential for *C. neoformans* growth at 37°C and for its virulence (1, 21, 35). Calcineurin consists of two subunits, the catalytic A (Cna1) and the regulatory B (Cb1) subunits, both of which are necessary for enzymatic function and survival of *C. neoformans* at 37°C (1, 12, 35). Studies of both pathogenic and nonpathogenic fungi have revealed important roles for calcineurin in myriad physiological processes, including morphogenesis, cell cycle progression, cytokinesis, septation, hyphal elongation during mating, cell wall biogenesis, and iron homeostasis (7, 8, 15, 16, 18, 28, 30, 40). The calcineurin signaling pathway is the target of the immunosuppressive drugs cyclosporine and FK506 (26). While the importance of calcineurin in fungal virulence has been established, its downstream targets in *C. neoformans* remain largely unknown (6).

The *CTS1* gene (calcineurin temperature suppressor 1) was identified in a multicopy suppressor screen of a calcineurin mutant of *C. neoformans* (11). Cts1 contains a C2 domain, which binds phospholipids and is found in a number of eukaryotic proteins involved in membrane trafficking, generation of lipid second messengers, activation of GTPases, and control of protein phosphorylation (32, 41). Fox et al. previously reported that overexpression of Cts1 restores growth at 37°C in *cna1Δ* and *cnb1Δ* calcineurin mutant strains and confers resistance to FK506 and cyclosporine at 37°C in wild-type (WT) cells (11). Like the *cna1Δ* mutant, the *cts1Δ* mutant is inviable at 37°C and avirulent in a murine model of cryptococcosis. Deletion of *CTS1* also confers defects in septation during vegetative growth and in hyphal elongation during mating. Transcription of *CTS1* is increased in the *cna1Δ* mutant, and the *cts1Δ cna1Δ* double mutant is synthetically lethal, suggesting that Cts1 and calcineurin operate in parallel pathways with shared functions in high-temperature growth and virulence.

However, this does not rule out the possibility that Cts1 and calcineurin cooperate in certain processes necessary for survival at 37°C. Our studies sought to elucidate the interconnections between Cts1 and the calcineurin signaling pathway, in-
TABLE 1. Strains and plasmids used in this study

| Strain or plasmid | Genotype | Source or reference |
|------------------|----------|---------------------|
| Strains          |          |                     |
| H99              | α        | 39                  |
| KN99             | a        | 34                  |
| EA04             | α cts1::NAT | This study         |
| EA83             | a cts1::NEO | This study         |
| EA11             | a GFP-CTS1::NAT | This study     |
| EA93             | a cts1::NEO GFP-CTS1::NAT | This study |
| EA124            | a cts1::NEO GFP-C2::NAT | This study |
| EA140A           | a cts1::NEO GFP-CTS1C2A::NAT | This study |
| LK274            | a GFP-CTS1::NAT mCH-MYO1::HYG | This study |
| LK278B           | a cts1::NAT mCH-MYO1::HYG | This study |
| LK227            | a GFP-CTS1::NAT mCH-RAB5::NEO | This study |
| LK243            | α mCH-CTS1-NEO GFP-CNA1::NAT | This study |
| EA132            | α cts1::NAT mCH-CNA1::NEO | This study |
| EA138            | α mCH-CTS1-NEO GFP-DCP1::NAT | This study |
| EA130            | a GFP-CTS1::NAT mCH-PUB1::NEO | This study |
| LK221            | α GFP-CNS5::NAT | 23                  |
| Plasmids         |          |                     |
| pLKB44B          | GFP-CTS1::NAT | This study         |
| pLKB50           | mCH-CTS1::NEO | This study         |
| pLKB93           | GFP-C2::NAT | This study         |
| pLKB94           | GFP-CTS1C2A::NAT | This study |
| pLKB77           | mCH-MYO1::HYG | This study         |
| pLKB51           | mCH-RAB5::NEO | This study         |
| pLKB39           | GFP-CNA1::NAT | 22                  |
| pLKB60           | mCH-CNA1::NEO | 22                  |
| pLKB87           | mCH-PUB1::NEO | 22                  |
| pWX11            | GFP-DCP1::NAT | 47                  |

Materials and Methods

Strains, medium, and growth conditions. All C. neoformans strains used in this study are derived from the congeneric serotype A (Cryptococcus neoformans var. grubii) strains H99 (MATa) and KN99 (MATa). Table S1 lists the strains and plasmids used in this study. Unless otherwise indicated, strains were grown on standard yeast medium.

Disruption of the CTS1 gene. Table S1 in the supplemental material includes primer sequences used for generating deletions and fluorescent protein chimeras. The 5' and 3' regions of CTS1 were amplified from H99α or KN99α genomic DNA, and the dominant selectable markers NAT (nourseothricin) and NEO (G418) were amplified from plasmids pNATSTM4209 and pJAF1 (14), respectively. The CTS1 gene replacement cassettes were generated by overlap PCR as previously described (9). The cassettes were then precipitated onto gold microcarrier beads, and strain H99α or KN99α was transformed biolistically as previously described (10). Stable transformants were selected on YPD medium containing nourseothricin or G418. To screen for cts1Δ mutants, diagnostic PCR was performed. Positive transformants were further confirmed by Southern blotting.

Generation of fluorescent protein chimeras. Primers used to generate fluorescent protein chimeras are listed in Table S1 in the supplemental material. To generate a strain expressing GFP-Cts1, a plasmid encoding GFP-CTs1 and containing a NAT resistance gene was engineered. The CTS1 open reading frame (ORF) together with 139 bp of the 5'-untranslated region (3'-UTR) (containing the flanking restriction sites for BamHI) was amplified from strain H99α digested with BamHI, and cloned into BamHI-digested and calf intestinal alkaline phosphatase (CIP)-treated plasmid pCN19 (kindly provided by Conrie Nichols and Andrew Alspaugh, Duke University). The resulting plasmid, pLKB44, expresses GFP-Cts1 under the control of a constitutive histone promoter. Strain KN99α or the cts1Δ strain EA83 was transformed biolistically with plasmid pLKB44 as described previously (10), and positive clones were screened based on the presence of a fluorescence signal. Positive transformants were further confirmed by Western blotting.

To generate a strain expressing GFP-C2, a sequence containing a stop codon followed by the 3'-UTR was ligated at an Xmal cleavage site within the CTS1 ORF in the plasmid pLKB44. This resulted in a chimera where green fluorescent protein (GFP) was followed by the first 265 amino acids (aa) of Cts1 (161 aa of the predicted C2 domain plus ~100 aa of downstream sequence). The cts1Δ strain EA83 was transformed biolistically with the resulting plasmid, pLKB93. Positive clones were screened based on the presence of a fluorescence signal and further confirmed by Western blotting.

To generate transformants expressing mCherry N-terminal fusion chimeras, plasmids pLKB49 (NEO) and pLKB55 (HYG) were used as described previously (22).

Microscopy. For imaging of yeast cells, ~0.5 μl of cell suspension was placed on a 2% agar complete medium patch on the slide and covered with a coverslip.

For the FM4-64 experiments, a working concentration of 80 μM was used (from a 16 mM stock). In pulse-chase experiments, cells were pulsed with FM4-64 on ice, washed, and analyzed immediately under a microscope. Images were taken every 5 min. Strain LK221, expressing the putative septin GFP-Cns5 (23), was used as a wild-type control with a GFP signal. LK221 grows as well as the wild type, and FM4-64 dynamics in this strain were similar to those of the H99 wild-type strain.

Bright-field, differential interference contrast (DIC), and fluorescence images were captured with either a Zeiss Axio Scope 2 Plus fluorescence microscope (Carl Zeiss, Thornwood, NY) equipped with an AxioCam MRm digital camera or a Zeiss Axio Scope microscope equipped with an Orca cooled charge-coupled device camera (Hamamatsu, Bridgewater, NJ) and interfaced with MetaMorph software (Universal Imaging, Silver Spring, MD). Images were processed using Adobe Photoshop.

Immunoblotting. Cells expressing GFP-Cts1 were grown overnight at room temperature in liquid yeast extract-peptone-dextrose (YPD). Cultures were refreshed to the same concentration after 60 min (OD600nm of ~0.3) and grown at room temperature until reaching an OD600 of ~0.7. At this point, each of the cultures was divided into four samples, which were subjected to the following four different conditions for 1 h: 24°C, 24°C plus FK506 treatment, 37°C, and 37°C plus FK506 treatment. In samples containing FK506, 1 μg/ml FK506 was added.

Approximately 1 × 107 cells were resuspended in 225 μl of pronase buffer (25 mM Tris-HCl, pH 7.5, 1.4 M sorbitol, 20 mM NaCl, and 2 mM MgCl2). Tri-chloroacetic acid (TCA) was added to a final concentration of 17% (vol/vol) and samples were stored at ~80°C. Cells were homogenized by vortexing with glass beads at 4°C for 10 min. The lysates were collected, and the beads were washed twice with 5% TCA to recover the remaining protein lysates. Precipitated proteins were collected by centrifugation at 4°C. Pellets were dried and resuspended in approximately 30 μl of lysis buffer (40 mM Tris-HCl, pH 6.8, 8.5 M urea, 5% SDS, 0.1 mM EDTA, 143 mM β-mercaptoethanol, and 0.4 mg/ml bromophenol blue). Residual TCA was neutralized by the addition of 2 M unlabeled Tris base. Prior to SDS-PAGE, samples were heated for 2 min at 42°C and then centrifuged at 14,000 rpm, and supernatants were loaded onto 4 to 20% polyacrylamide gels.

To detect GFP fusion proteins, an anti-GFP polyclonal antibody (Santa Cruz...
Biotech, Santa Cruz, CA) was used at a 1:1,000 dilution. The secondary antibody was an anti-rabbit horseradish peroxidase (HRP)-conjugated antibody used at a 1:10,000 dilution. A PSTAIR antibody (Abcam, Cambridge, MA) was used at a 1:2,000 dilution as a loading control.

**CIP assay.** An overnight culture of cells expressing GFP-Cts1 was diluted to an OD$_{600}$ of ~0.3 and grown until reaching an OD$_{600}$ of ~0.7. FK506 was added to a concentration of 1 µg/ml, and cells were shifted to 37°C for 1 h. One hundred milliliters of the culture was then harvested. All subsequent steps were performed at 4°C. Cells were collected by centrifugation and resuspended in 3 volumes of ice-cold breaking buffer (10 mM Tris, 150 mM NaCl, 0.5 mM EDTA, pH 7.5) containing 1 mM phenylmethylsulfonyl fluoride (PMSF) and a protease inhibitor cocktail. An equivalent volume of glass beads was added, and cells were shaken on a bead beater eight times for 90 s each, with 2-min interludes. Cell lysates were transferred to fresh tubes. Four volumes of breaking buffer was used to wash the beads. Lysates were cleared by centrifugation for 20 min at 14,000 rpm (4°C), and supernatants were transferred to fresh tubes. Lysates were incubated for 1 h with 90 µl of the antibody-conjugated protein A agarose beads (2 µl of anti-GFP antibody). The beads were then pelleted and washed four times with the breaking buffer and once with CIP buffer (50 mM Tris-HCl, pH 8.0, 1 mM MgCl$_2$, 0.1 mM ZnCl$_2$) containing 1 mM PMSF and protease inhibitor cocktail.

After immunoprecipitation, beads were resuspended in 30 µl of CIP buffer. Reaction mixtures were then incubated with or without the addition of 15 U of CIP (NEB) at 37°C for 15 min. A parallel reaction was carried out in phosphate buffer containing CIP and 10 mM sodium orthovanadate as a phosphatase inhibitor. The beads were then resuspended in an equivalent volume of 2× loading buffer, boiled for 5 min, and electrophoresed in polyacrylamide-SDS gels.

**Immunoprecipitation.** Cells coexpressing mCherry-Cts1 and GFP-Cna1 were grown overnight in liquid YPD, diluted in the morning to a starting OD$_{600}$ of ~0.3, and grown until reaching an OD$_{600}$ of ~0.7. The same procedure was also used for the negative control, in which cells expressed only GFP-Cna1. At this point, each of the cultures was divided into two samples, which were grown at either 24°C or 37°C. All subsequent steps were performed at 4°C. Cells were collected by centrifugation and resuspended in 3 volumes of ice-cold breaking buffer (10 mM Tris, 150 mM NaCl, 0.5 mM EDTA, pH 7.5) containing 1 mM PMSF and a protease inhibitor cocktail (Roche). An equivalent volume of glass beads was added, and cells were shaken on a bead beater 10 times for 90 s each, with 2-min periods of cooling. Cell lysates were transferred to fresh tubes. Four volumes of breaking buffer was used to wash the beads. Lysates were cleared by centrifugation for 20 min at 14,000 rpm (4°C), and supernatants were transferred to fresh tubes.

RFP-Trap beads (Chromotek GmbH) were then added to each of the samples, and mixtures were tumbled at 4°C for 1 h. The beads were then pelleted and washed four times with the breaking buffer. After immunoprecipitation, the beads were resuspended in an equivalent volume of 2× loading buffer, boiled for 5 min, and electrophoresed in polyacrylamide-SDS gels.

To detect mCherry-Cts1, an anti-DoRed polyclonal antibody (Invitrogen) was used at a 1:1,000 dilution. The secondary antibody was an anti-rabbit HRP-conjugated antibody used at a 1:10,000 dilution.

Other methods. Analysis of cell viability, 10-fold serial dilutions from overnight liquid cultures were performed, and 2 µl of each dilution was spotted on an appropriate medium as indicated in the figure legends. The largest number of spotted cells was 10⁵ cells.

To perform matings of C. neoformans, cells of opposite mating types were mixed in water, spotted on V8 or MS agar medium, and cultured at 25°C in the dark. Formation of hyphae, basidia, and chains of basidiospores was examined by light microscopy.

**RESULTS**

**Phenotype of the serotype A cts1Δ mutant strain.** Deletion of the CTS1 gene was previously shown to cause inviability at 37°C and to confer defects in septation during vegetative growth and in hyphal elongation during mating in serotype D strains (Cryptococcus neoformans var. neoformans) (11). Our studies examined the role of Cts1 in strains of serotype A (Cryptococcus neoformans var. grubii), which is the predominant serotype among clinical isolates (31, 45). Given that the serotype A and serotype D lineages have considerable genetic and genomic differences, we examined the phenotype of the serotype A cts1Δ mutant strain to gain insights into its function in this variety (13).

The C2 domain of Cts1 was previously shown to be essential for high-temperature growth, septation, and phospholipid binding in serotype D strains (11). However, the phenotype and viability at 37°C of a strain expressing only the C2 domain of Cts1, which would provide insights into whether the C2 domain of Cts1 alone is sufficient to complement the growth defects of the cts1Δ mutant, remained unknown. In addition to examining the phenotype of the cts1Δ mutant, we therefore also sought to examine the phenotypes of strains lacking the C2 domain of Cts1 or expressing only the C2 domain of Cts1.

The CTS1 gene was disrupted by homologous recombination with a cassette containing either the nourseothricin or neomycin resistance gene. Mutation of the CTS1 gene was confirmed by PCR and Southern blot analysis (data not shown). The cts1Δ strain was complemented with CTS1 fused to the GFP gene at the N terminus and ectopically expressed under the control of a constitutive histone H3 promoter. Similarly, the C2 domain of Cts1 and a truncation allele lacking the C2 domain were expressed ectopically from the histone H3 promoter as GFP fusions in the cts1Δ background (Fig. 1A). Sequencing and Western blot analysis were used to confirm that these fusion proteins were stably expressed (see Fig. S1 in the supplemental material). However, we noted that a small fraction (5 to 10%) of cells expressing mutant versions of Cts1 fused to GFP did not show a fluorescence signal based on microscopic observations (data not shown).

Growth of the cts1Δ strain was largely affected at 37°C in a spot dilution growth assay, consistent with the phenotype previously reported for serotype D (11) (Fig. 1B). GFP-Cts1 improved growth in the cts1Δ background, indicating that the GFP tag did not significantly affect the function of Cts1. While the cts1Δ strain exhibited partial growth at 37°C, the cts1Δ GFP-C2 and cts1Δ GFP-Cts1Δ2 strains did not grow at 37°C, which suggests that both the C2 domain and the C-terminal domain of Cts1 are essential for high-temperature growth and that expression of these constructs may have a negative effect on cell growth at 37°C. Strikingly, the cts1Δ GFP-Cts1Δ2 strain also grew slower than the cts1Δ strain at 24°C, suggesting that expression of GFP-Cts1Δ2 may have a negative effect on cell growth even at 24°C.

The serotype A cts1Δ strain did not grow in a spot dilution growth assay on medium containing the calcineurin inhibitor FK506 at 24°C (Fig. 1B). This drug-imposed phenotype indicates that Cts1 and calcineurin likely function in parallel or branched pathways in serotype A, which is consistent with previous findings on serotype D (11). Strikingly, the GFP-C2 allele was able to partially complement the growth defect of the cts1Δ strain in the presence of FK506 at 24°C, although significantly less than GFP-Cts1. The cts1Δ GFP-Cts1Δ2 strain was not viable in the presence of FK506 at 24°C, which suggests that the fragment containing the C2 domain may be essential for survival in the absence of calcineurin at 24°C.
buds, multiple buds, and/or misshapen buds (Fig. 1C and D). The cytokinesis defect was rescued by the expression of GFP-Cts1, as the morphology of cts1/H9004 GFP-Cts1 cells was similar to that of WT GFP-Cts1 cells (Fig. 1C; see Fig. S2 in the supplemental material). Both the cts1/H9004 GFP-C2 and cts1/H9004 GFP-Cts1C2/H9004 strains also exhibited a cytokinesis defect, indicating that neither fragment is able to fully complement the cts1/H9004 mutant phenotype. However, the cts1/H9004 GFP-C2 strain had a less severe phenotype than the cts1/H9004 GFP-Cts1C2/H9004 strain, as a smaller percentage of aberrant cells (defined as cells with unusually large buds, multiple buds, and/or misshapen buds) was observed (Fig. 1C and D). The morphological defect of the cts1/H9004 GFP-Cts1C2/H9004 strain was also more severe than that of the cts1/H9004 strain, which further supports the hypothesis that expression of GFP-Cts1C2/H9004 has a negative effect on cell morphology.

We also examined whether the serotype A cts1Δ strain exhibits mating defects similar to those observed in serotype D (Fig. 1E). No defects were observed when either the α cts1::NAT or a cts1::NEO strain was cocultured with wild-type tester strains in a unilateral cross on MS medium. However, no hyphae, basidia, or spores were observed when α cts1::NAT and a cts1::NEO mutant strains were cocultured in a bilateral cross on MS medium, which is consistent with previous findings in serotype D (11). To determine whether Cts1 is necessary for cell-cell fusion, the mating patches were resuspended in distilled water and plated on nourseothricin and G418 double-selection medium. No doubly resistant isolates were obtained, indicating that Cts1 is necessary for cell fusion during mating (data not shown). It is important that while calcineurin is essential for production of recombinant basidiospores, it is not necessary for cell fusion, suggesting that involvement in cell fusion is a calcineurin-independent function of Cts1 (7).

Cts1 localizes to cytoplasmic puncta and appears transiently at the mother bud neck during cytokinesis. To gain further insights into the function of Cts1, localization of GFP-Cts1 was examined. In some large budded cells, a single ring corresponding to GFP-Cts1 was observed at the mother bud neck (Fig. 2). The fact that the vast majority of cells did not show localization of GFP-Cts1 to the mother bud neck suggested that this localization might be transient. To address this question, we performed time-lapse analysis of cells expressing GFP-Cts1 (Fig. 2A). GFP-Cts1 underwent constriction at the mother bud neck similar to that of Myo1, which is part of the actomyosin ring and plays a critical role in cytokinesis (2). GFP-Cts1 constricted as a single ring and later appeared on
both sides of the neck as either two single spots or a double ring. To examine whether Cts1 may also be a part of the actomyosin ring, mCherry-Myo1 was coexpressed with GFP-Cts1. In large budded cells, the constriction of GFP-Cts1 closely followed that of mCherry-Myo1 but occurred later, suggesting that Cts1 is not part of the actomyosin ring (Fig. 2B). We also examined the possibility that Cts1 may be necessary for Myo1 function by expressing mCherry-Myo1 in a cts1/H9004 mutant strain. mCherry-Myo1 appearance at the bud neck during cytokinesis was not affected by the absence of CTS1 (data not shown).

In addition to the bud neck localization, GFP-Cts1 was also observed at cytoplasmic puncta in all cells. At 24°C, GFP-Cts1 localized to an average of 2.9 cytoplasmic puncta per cell in the cts1/H9004 background (Fig. 3A and B). Because CTS1 is essential for high-temperature growth, we also examined whether Cts1 undergoes a change in localization during high-temperature stress. The cts1/H9004 GFP-Cts1 strain was incubated on a micro-
scope slide that was placed on a heating block at 37°C. While GFP-Cts1 still localized to cytoplasmic puncta and to the mother bud neck in some large budded cells, the number of cytoplasmic puncta at which GFP-Cts1 localized increased from an average of 2.9 per cell to 4.7 per cell after only 30 min of incubation at 37°C (Fig. 3A and B). This suggests that the function of Cts1 at cytoplasmic puncta may be upregulated and become more important during high-temperature stress response.

We also examined whether the C2 domain of Cts1 is either necessary or sufficient for localization to cytoplasmic puncta and the mother bud neck (Fig. 3A). In the cts1Δ GFP-Cts1C2Δ strain, the GFP tag signal showed localization to puncta and the mother bud neck, similar to that of the cts1Δ GFP-Cts1 strain. However, the average number of GFP-Cts1C2Δ cytoplasmic puncta was ~2-fold higher at 24°C and ~1.2-fold higher at 37°C than that of GFP-Cts1 cytoplasmic puncta (Fig. 3B). On the other hand, the cts1Δ GFP-C2 strain exhibited mainly diffuse cytoplasmic localization, with only a few cells showing localization to puncta. However, the average number of GFP-C2 puncta per cell increased when cells were shifted to 37°C. Localization of GFP-C2 to the mother bud neck was not observed. This indicates that the C2 domain is neither necessary nor sufficient for localization to cytoplasmic puncta or the mother bud neck. Proper localization, however, is not sufficient for Cts1 functions to support high-temperature growth, as the growth of the cts1Δ GFP-Cts1C2Δ strain was clearly affected at 37°C, despite the ability of the Cts1C2Δ protein to localize to cytoplasmic puncta and the mother bud neck.

Cts1 colocalizes with the endosomal marker FM4-64 and may play a role in endocytic trafficking. The localization of GFP-Cts1 to both cytoplasmic puncta and the mother bud neck in large budded cells suggested two distinct functions for Cts1. Previous work implicated a role for Cts1 in septation, which is consistent with localization of GFP-Cts1 to the mother bud neck (11). We also further explored the cytoplasmic localization of Cts1 to determine the subcellular compartments or structures to which Cts1 localizes, which would provide insights into other potential roles of Cts1. Colocalization was not observed when cells expressing GFP-Cts1 were stained with MitoTracker (data not shown), which indicates that Cts1 puncta do not correspond to mitochondria.

To determine whether the cytoplasmic puncta of Cts1 represent endosomes or vacuolar membranes, cells expressing GFP-Cts1 were pulsed on ice with the endocytic tracker dye FM4-64, incubated at 30°C, and analyzed every 15 min. After 45 min of incubation at 30°C, colocalization was observed at cytoplasmic puncta that may represent late endosomes (Fig. 4A). This indicates that Cts1 is localized to and may be involved in the endocytic pathway. To further explore this possibility, GFP-Cts1 was coexpressed with the mCherry-tagged early endosomal marker Rab5. Although colocalization was not observed in the majority of cells, the GFP and mCherry signals did overlap in some cells (out of 50 cells, 13 showed at least one punctum exhibiting colocalization) (Fig. 4B). This further supports the hypothesis that Cts1 may play a role in endocytosis.

To further investigate a possible role for Cts1 in endocytosis, we examined the FM4-64 staining pattern in the cts1Δ mutant strain. A vacuolar staining pattern was observed in the cts1Δ mutant, whereas the wild type showed punctate staining (Fig. 4C). The punctate staining pattern of FM4-64 observed in wild-type cells is strikingly different from that seen in Saccharomyces cerevisiae, which typically shows sustained vacuolar staining. In fact, the staining in cts1Δ cells more closely resembled that in S. cerevisiae cells. To further examine this distinct phenotype, time-lapse analysis of FM4-64 localization was performed in a pulse-chase experiment. The cts1Δ mutant and a wild-type GFP-expressing strain, LK221, were mixed at a 1:1 ratio and examined simultaneously on the same slide to ensure identical conditions. The GFP signal was used to distinguish between the wild type and the cts1Δ mutant (Fig. 4D). Cells were pulsed with FM4-64 on ice, washed, and immediately analyzed by time-lapse microscopy to test the progression of endocytosis. In both wild-type and mutant cells, FM4-64 reached the vacuole, similar to the staining described for S. cerevisiae. However, in contrast to the case in S. cerevisiae, FM4-64 eventually disappeared from the vacuolar membrane. In the GFP-expressing wild-type strain, the percentage of cells showing vacuolar staining of FM4-64 increased initially and then declined to ~6% after 60 min. In the cts1Δ mutant, the percentage of cells showing vacuolar staining increased initially, but the decline was delayed compared to that of the wild type and the proportion of cells showing a vacuolar signal remained at 30% after 30 min, compared to 13% of the wild type (Fig. 4D). We eliminated the possibility that this difference was due to the GFP tag by examining the FM4-64 staining pattern in a 1:1 culture of wild-type GFP-expressing and non-GFP-expressing cells. Both wild-type strains showed similar dynamics of FM4-64 progression through endocytosis (Fig. 4D). These findings suggest that Cts1 plays a role in endocytic trafficking and may be involved specifically in recycling of the vacuolar membrane.

mCherry-Cts1 colocalizes with GFP-Cna1 at sites of mRNA processing. We previously reported that the catalytic subunit of calcineurin, GFP-Cna1, undergoes a relocalization to cytoplasmic puncta during high-temperature stress in C. neoformans (22). Because GFP-Cts1 also localizes to cytoplasmic puncta at 37°C, we examined whether calcineurin and Cts1 colocalize, which would be consistent with concerted functions in processes necessary for high-temperature growth. Cts1 was tagged with mCherry at the N terminus and expressed ectopically under the control of a constitutive GPD1 promoter. GFP-Cna1 was coexpressed with mCherry-Cts1, and strikingly, colocalization was observed at 37°C but not at 24°C (Fig. 5A). While the average number of puncta corresponding to mCherry-Cts1 was greater than the number corresponding to GFP-Cna1, colocalization was observed at every cytoplasmic punctum at which GFP-Cna1 was present.

To test whether Cts1 localization to cytoplasmic puncta is dependent on calcineurin catalytic activity, cells expressing GFP-Cts1 were treated with the calcineurin inhibitor FK506. GFP-Cts1 localization did not change significantly at either 24°C or 37°C, suggesting that calcineurin catalytic activity is not necessary for the localization of Cts1 (data not shown). We also examined whether calcineurin localization depended on Cts1 by expressing GFP-Cna1 in the cts1Δ mutant strain. Calcineurin localization at both 24°C and 37°C was similar to that previously reported for the wild type (22), indicating that cal-
Cineurin localization is not dependent on Cts1 (data not shown). We also previously reported that the P-body component Dcp1 and the stress granule constituent Pub1 colocalize with Cna1 at 37°C, suggesting a role for calcineurin in regulation of protein synthesis (22). To test whether Cts1 puncta also colocalize with these proteins, mCherry-Cts1 was coexpressed with GFP-Dcp1 and GFP-Cts1 was coexpressed with mCherry-Pub1. GFP-Dcp1 and mCherry-Cts1 colocalized at 24°C, but not at all cytoplasmic puncta (Fig. 5B). On the other hand, mCherry-Cts1 largely colocalized with GFP-Dcp1 and GFP-Cts1 largely colocalized with mCherry-Pub1 at 37°C (Fig. 5B and C). At 24°C, however, mCherry-Pub1 showed diffuse cytoplasmic localization, and colocalization with GFP-Cts1 was not observed. These findings suggest a potential role for Cts1 in regulation of mRNA processing at P-bodies and stress granules, especially during the high-temperature stress response.

Cts1 is a phosphoprotein and is hyperphosphorylated in the presence of FK506. Colocalization of mCherry-Cts1 and GFP-Cna1 at 37°C suggested that Cts1 and calcineurin function coordinately in processes necessary for high-temperature growth. To further examine the interconnection between Cts1 and the calcineurin signaling pathway, we investigated whether Cts1 may be a substrate of the protein phosphatase calcineurin. Cells expressing GFP-Cts1 were grown at 24°C and 37°C in the presence and absence of the calcineurin inhibitor FK506. GFP-Cts1 was subsequently detected by Western blot analysis, which revealed an additional band at 37°C with a slower electrophoretic mobility than at 24°C (Fig. 6A). Strikingly, the signal corresponding to the higher-molecular-weight band of GFP-Cts1 was even more abundant in the presence of FK506 at 37°C.

To determine whether the shift to a higher molecular weight was due to phosphorylation, cells expressing GFP-Cts1 were grown at 37°C in the presence of FK506 and immunoprecipitated using GFP-Trap beads. The beads were subsequently treated with CIP. Western blot analysis revealed a faster electrophoretic mobility of GFP-Cts1 for the sample incubated with CIP (Fig. 6B). On the other hand, the sample incubated with both CIP and sodium orthovanadate, a phosphatase in-
hibitor, did not show faster electrophoretic mobility. These data indicate that Cts1 is phosphorylated during high-temperature stress and is hyperphosphorylated in the presence of FK506, suggesting that Cts1 may be dephosphorylated in a calcineurin-dependent manner.

**mCherry-Cts1 and GFP-Cna1 coimmunoprecipitate at 37°C.** Colocalization of mCherry-Cts1 and GFP-Cna1 at 37°C and the hyperphosphorylated state of GFP-Cts1 in the presence of FK506 at 37°C suggested that Cts1 may be a substrate of calcineurin. To further test this hypothesis, we examined whether Cts1 and calcineurin are part of the same complex in vivo at either 24°C or 37°C. Cells coexpressing mCherry-Cts1 and GFP-Cna1 were grown at either 24°C or 37°C. mCherry-Cts1 and any interacting proteins were subsequently immunoprecipitated from cell lysates by using RFP-Trap beads. Strikingly, GFP-Cna1 was detected on the immunoblot for both the 24°C and 37°C immunoprecipitation samples, with a significantly greater abundance detected at 37°C (Fig. 6C). The abundance of mCherry-Cts1, on the other hand, was roughly equivalent in both samples, indicating that greater complex formation between GFP-Cna1 and mCherry-Cts1 was detected at both 24°C and 37°C and was significantly more pronounced at 37°C. To better display the input, a shorter exposure is shown in the lower left panel. Similarly, a longer exposure of the immunoprecipitation is shown in the lower right panel to illustrate the difference between 24 and 37°C.

**FIG. 6.** Cts1 appears to be a substrate of calcineurin during high-temperature stress response. (A) Western blot analysis of GFP-Cts1 showing slower electrophoretic mobility at 37°C, which was more pronounced in the presence of the calcineurin inhibitor FK506. (B) CIP treatment of immunoprecipitated GFP-Cts1 confirmed that the slower electrophoretic mobility at 37°C in panel A was due to phosphorylation. (C) mCherry-Cts1 was immunoprecipitated using RFP-Trap beads in samples grown at either 24°C or 37°C. Coimmunoprecipitation of GFP-Cna1 and mCherry-Cts1 was detected at both 24°C and 37°C and was significantly more pronounced at 37°C. To better display the input, a shorter exposure is shown in the lower left panel. Similarly, a longer exposure of the immunoprecipitation is shown in the lower right panel to illustrate the difference between 24 and 37°C.

**FIG. 5.** Cts1 and calcineurin colocalize at sites of mRNA processing during high-temperature stress. (A) Cells coexpressing mCherry-Cts1 and GFP-Cna1 were examined at both 24°C and 37°C. At 24°C, GFP-Cna1 showed diffuse cytoplasmic localization and colocalization with mCherry-Cts1 was not observed. Strikingly, at 37°C, calcineurin underwent a relocalization to cytoplasmic puncta, where it largely colocalized with mCherry-Cts1. (B) mCherry-Cts1 was coexpressed with the P-body component GFP-Dcp1 to test for colocalization at 24°C and 37°C. Cts1 and Dcp1 mostly colocalized at 37°C. However, significant colocalization was not observed at 24°C. While mCherry-Cts1 and GFP-Dcp1 did colocalize at some puncta (arrow 1), only partial colocalization was observed at others (arrow 2). There were also puncta where only GFP-Dcp1 appeared (arrow 3) and some where only mCherry-Cts1 localized (arrow 4). (C) GFP-Cts1 was coexpressed with the mCherry-tagged stress granule component Pub1. At 24°C, mCherry-Pub1 exhibited diffuse cytoplasmic localization, and colocalization with GFP-Cts1 was not observed. However, at 37°C, mCherry-Pub1 localized to cytoplasmic puncta that mostly colocalized with GFP-Cts1 (arrow).

**DISCUSSION**

The importance of calcineurin phosphatase activity in eukaryotic transcriptional regulation during stress has been well established. Important targets of calcineurin include members of the nuclear factor of activated T cell (NFAT) family of transcription factors, which activate immune responses in multicellular eukaryotes (20). In the budding yeast *Saccharomyces cerevisiae*, dephosphorylation of Crz1 by calcineurin leads to elevated transcription of more than 160 genes that promote remodeling of the cell surface in response to stress (8). Importantly, no clear functional homologue of the *CRZ1* gene has been identified in *C. neoformans* serotype A or D, suggesting...
that the effects of calcineurin in *C. neoformans* could be mediated by different transcription factors or may be partly or entirely posttranscriptional (24).

Overexpression of the C2 domain protein Cts1 was previously shown to restore growth at 37°C in a calcineurin cna1Δ mutant of *C. neoformans*, which suggested that Cts1 may be a substrate and possibly an effector of calcineurin during high-temperature stress (11). Here we report three findings that support this hypothesis: (i) mCherry-Cts1 and GFP-Cna1 colocalize specifically during thermal stress at 37°C; (ii) Cts1 is hyperphosphorylated in the presence of the calcineurin inhibitor FK506 at 37°C, which suggests that Cts1 is dephosphorylated in a calcineurin-dependent manner; and (iii) mCherry-Cts1 and GFP-Cna1 coimmunoprecipitate, with stronger complex formation at 37°C than at 24°C. It is important that Cts1 also contains the LAVP calcineurin-binding motif found in NFATc1 and NFATc4, as well as the LAPP calcineurin-binding motif found in the calcineurin regulatory protein RCAN1 (25, 27, 29, 37, 43) (Fig. 1A). Taken together, these findings reveal a novel posttranscriptional role of calcineurin during high-temperature stress responses in *C. neoformans*.

Cts1 was first isolated and described for serotype D, so in this study we examined the function of Cts1 in serotype A. Interestingly, the *CTS1* ORF in serotype A lacks the sequence for a putative leucine zipper motif that was previously described for serotype D (11). This motif was dispensable for growth at 37°C but was found to be essential for virulence, proper hyphal elongation, and viability in the absence of calcineurin function in serotype D (11). The absence of the leucine zipper motif in serotype A extends previous findings indicating that this domain is not essential for growth at 37°C and suggests that the effects of the C-terminal truncation of Cts1 in serotype D may be due to a lack of other important motifs within the C terminus of Cts1.

What is the role of Cts1 in *C. neoformans* high-temperature growth and virulence? It was previously reported that deletion of *CTS1* confers defects in septation during vegetative growth and in hyphal elongation during mating (11). Here we examined the localization of Cts1 to gain further insights into its function. GFP-Cts1 localizes to the mother bud neck as a single ring in large budded cells. The constriction of this ring closely follows that of the actomyosin ring component Myo1. While the Cts1 dynamics do not completely match those of the actomyosin ring, it is still possible that at some point during the cell cycle, Cts1 associates with the actomyosin ring. The double ring of GFP-Cts1 after it constricts suggests a possible association with the septin double ring, but testing this will require further studies. Our localization studies support a potential role for Cts1 in cytokinesis. A recent study in the fission yeast *Schizosaccharomyces pombe* provides some clues to the potential role of Cts1 in cytokinesis (42). The *S. pombe* C2 domain protein Fic1 was identified and shown to exhibit homology to both Cts1 and the *S. cerevisiae* protein Inm1 (42). Fic1 adds structural integrity to the contractile ring during cytokinesis and binds to the SH3 domain of Cdc15, which participates in cellular processes that bridge the plasma membrane and cytoskeleton. It is possible that Cts1 carries out similar functions during cytokinesis in *C. neoformans*. Importantly, Cts1 is rich in prolines, which is consistent with a possible interaction with SH3 domains (33). Future studies should further explore the specific role of Cts1 in cytokinesis.

GFP-Cts1 also localizes to cytoplasmic puncta that colocalize with the endocytic tracker FM4-64 at late endosomes. Strikingly, compared to the wild type, the cts1Δ strain showed a higher percentage of cells with vacuolar FM4-64 staining during progression through endocytosis, suggesting that Cts1 may play a role in vacuolar membrane trafficking. This is consistent with previous studies that have reported involvement of C2 domain proteins, including synaptogamin I and raphilin-3A, in membrane trafficking (4, 46).

It is also unknown whether there are connections between the roles of Cts1 in membrane trafficking and cytokinesis. Schink and Bolker recently reported that the *Ustilago maydis* Cdc42-specific guanine nucleotide exchange factor Don1 localizes to fast-moving endosomal vesicles that accumulate at the site of septation during cytokinesis (44). Cts1 may have a similar function in the cytoplasmic puncta and at the mother bud neck, but further studies are necessary to investigate this hypothesis.

In addition to potential roles in endocytic trafficking and cytokinesis, Cts1 may also be involved in mRNA processing and regulation of protein translation during high-temperature stress. At 37°C, Cts1 colocalizes with the calcineurin catalytic subunit Cna1, the P-body constituent Dcp1, and the stress granule component Pub1. Colocalization of Cts1 and Cna1 at sites of mRNA processing suggests that Cts1 may also play a role in regulation of translation. Future studies should investigate this potential role.

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