The fungal Tup1 proteins function as global repressors which regulate a large number of genes associated with growth, morphological differentiation, and sexual and asexual reproduction. As a consequence, tup1 mutants are known to display numerous phenotypes (9, 19, 42). The deletion of TUP1 in Candida albicans results in constitutive filamentous growth with no budding yeast cells and is accompanied by loss of virulence (2, 32). In Penicillium marneffei, the only dimorphic species known in the genus Pencillium, deletion of the TUP1 homolog, tup4, confers reduced filamentation and abnormality in yeast morphogenesis (38). In the filamentous fungi Aspergillus nidulans and Neurospora crassa, deletion of the TUP1 homologs, rcoA and rco-1, respectively, severely affects growth and sexual and asexual reproduction (12, 46).

Cryptococcus neoformans is a bipolar heterothallic basidiomycetous yeast with two serotypes, A and D, and the function of Tup1 has been studied only for serotype D strains (26, 27). While disruption of TUP1 in strains of serotype D did not affect yeast or hyphal cell morphology, it resulted in mating-type-dependent differences, including temperature-dependent growth, sensitivity to 0.8 M KCl, and expression of genes in several other biological pathways (26). Most importantly, tup1Δ strains displayed a peptide-mediated quorum-sensing-like phenomenon in both mating types of serotype D strains which has not been reported for any other fungal species (27).

According to genome sequence data, the serotype A reference strain H99 shares 95% sequence identity with the serotype D reference strain JEC21 (29). However, serotype-specific differences between the two strains have been demonstrated in two major signaling pathways, the phosphorelay-Cpk1 mitogen-activated protein kinase and cyclic AMP (cAMP) (5, 13, 41, 47). In addition, the high-osmolarity glycerol (HOG) pathway also showed regulatory disparity between the two serotypes (1, 8). Since the regulation of peptide-mediated quorum sensing by TUP1 is reported only for serotype D strains, we sought to determine whether the deletion of TUP1 in serotype A strains would have similar consequences. Surprisingly, we found striking differences in the phenotypes manifested by tup1Δ strains of the two serotypes. We report here the serotype-specific differences in TUP1 regulation between A and D strains and the novel regulatory role of TUP1 in maintaining iron/copper homeostasis in C. neoformans.
of the locus was amplified with primers TND-C1 and TND-C2G418; the right
end of the locus was amplified with primers TND-DIG418 and TND-D2.
G418-A1 and G418-B2 were used to amplify the NEO (neomycin phosphotrans-
ferase II) selectable marker from the plasmid pJAF1 (a gift from J. Heitman) (see
table S1 in the supplemental material). The upstream and downstream
flanking regions of the TUP1 gene were amplified from the genomic DNA of
each strain using the same primers. The amplified products were gel purified and
used as templates to produce a 4.2-kb <i>tup1</i>-Δ deletion construct containing
the flanking regions of the TUP1 gene connected by the NEO gene. The linear
disruption cassette was then used to homologously integrate into the strains
by biolistic transformation (39). Transformants were screened to identify the <i>tup1</i>-Δ
strains by colony PCR. Deletion of <i>TUP1</i> was confirmed by Southern blot
hybridization (see Fig. S1 in the supplemental material).

To obtain the H99 <i>TUP1</i> gene, a 4.8-kb DNA fragment containing the 1.3-kb
flanking region on both sides was PCR amplified from H99 genomic DNA,
sequenced, and cloned into the pAI3 vector (a gift from J. Heitman) containing
the <i>NAT</i> selectable marker to obtain pHL110. pHL110 was linearized with Smal
and transformed into the H99 <i>tup1</i>-Δ strain by the biolistic method. PCR was used
to identify integrative transformants containing the intact <i>TUP1</i> gene, and South-
ern blot analysis was used to confirm the integration event (see Fig. S1 in the
supplemental material).

Preparation and analysis of nucleic acid. Isolation and analysis of genomic
DNA were carried out as described previously (4). For gene expression analysis,
overnight cultures of wild-type (H99) and <i>tup1</i>-Δ strains were refreshed and
grown in RPMI for 6 h. RNA was extracted from yeast cells using Trizol
(Invitrogen, Carlsbad, CA), treated with RNase-free DNase (Ambion, Austin,
TX) for the removal of genomic DNA, and purified with the RNaseasy MinElute
cleanup kit (Qiagen, Valencia, CA). cDNA was synthesized using a high-capacity
cDNA archive kit (Applied Biosystems, Foster City, CA) and used in real-time
reverse transcription-PCR (RT-PCR) with TaqMan universal PCR master mix
(Applied Biosystems, Foster City, CA). The primers used in RT-PCR are listed in Table S2 in the supple-
mental material. Data were normalized with actin levels and expressed as the
relative amount in the <i>tup1</i>-Δ strain compared to that in H99. In addition, the transcription
level of <i>CNAG_J0812.2</i> was normalized with γ-tubulin as an inter-
nal control.

Assays for mating, melanization, and capsule formation. For mating assays,
strains were grown on YEPD agar slants for 2 days. The cells of <i>MATa</i>
and <i>MATa</i> strains were mixed on V8 juice agar medium, incubated, and monitored
for evidence of mating. Melanin production was estimated after spotting serially
diluted yeast cells onto Trizol (Invitrogen, Carlsbad, CA, treated with RNase-free DNase (Ambion, Austin,
TX) for the removal of genomic DNA, and purified with the RNaseasy MinElute
cleanup kit (Qiagen, Valencia, CA) and used in real-time
reverse transcription-PCR (RT-PCR) with TaqMan universal PCR master mix
(Applied Biosystems, Foster City, CA). The primers used in RT-PCR are listed in Table S2 in the supple-
mental material. Data were normalized with actin levels and expressed as the
relative amount in the <i>tup1</i>-Δ strain compared to that in H99. In addition, the transcription
level of <i>CNAG_J0812.2</i> was normalized with γ-tubulin as an inter-
nal control.

Virulence study. Female BALB/c mice (6 to 8 weeks old) were infected via the
lateral tail vein with 0.2 ml of a suspension of each yeast strain (5 × 10<sup>5</sup>cells/ml) as
described previously (4), and the mortality was monitored. Kaplan-Meier anal-
ysis of survival was performed with JMP software for Macintosh (SAS Institute,
Cary, NC). To measure the growth rate of each strain in the brain, mice were
inoculated with yeast cells (10<sup>5</sup> cells) as described above, and then three mice per
yeast strain were sacrificed at several intervals after injection (at 2 days as
the starting point and at 6, 9, and 13 days postinfection). The brains were homoge-
nized with a mortar and pestle, diluted, and then plated onto YEPD agar.
Colonies were counted after 2 days of incubation at 30°C.

Spot assay. Exponentially growing cultures (optical density at 600 nm OD<sub>600</sub>
(0.5 to 1.0) were washed, resuspended in 0.9% NaCl and adjusted to an OD<sub>600</sub>
of 0.1 for the wild type or 0.2 for the <i>tup1</i>-Δ strain (to compensate for its low
growth rate). Adjusted cell suspensions were serially diluted, spotted onto the
indicated media, and incubated for 3 to 4 days at 30°C. Limited-iron medium
(LIM) was identical to chemically defined medium (34) except that the salts of
polyvalent metals were dissolved in Chelex-100-treated water (Bio-Rad), and
other components were purified by treating with Chelex-100. When more strin-
gent control of iron or copper concentration was needed, 0.056 mM ethylenedi-
amine-diacetic acid (EDDA) (Sigma-Aldrich) or 1 mM bathocuproine sulfonate
(BCS) (Sigma-Aldrich) was added to the medium, respectively. LIM-Fe was
prepared by adding 0.1 mM ferric EDDTA (Sigma-Aldrich EDFS) to LIM.

Microarray analysis. The previous microarray study with <i>TUP1</i> in a serotype
D strain was done with a mini-microarray since a whole-genome cryptococcus
array was not available at that time (26). Recently, a whole-genome array con-
taining 7,738 70-mer oligomers was constructed by an academic consortium at
the University of Washington, St. Louis. It has been shown that H99 and JEC21 share
95% identity in their genome sequences (29). Although arrays were de-
signated based on serotype D strain JEC21, the arrays can be useful to assess the
deletion effect of a specific gene in serotype A as long as the corresponding
serotype A wild-type control strain is employed as a reference. RNA was ex-
tracted from H99 and HL112 grown in RPMI liquid medium for 3 h and 6 h, and
microarray analysis was performed as described before (25). Two arrays were
used for each time point, and all the genes whose average expression was
affected by greater than twofold in the <i>tup1</i>-Δ strain compared with the wild-type strain
when grown for 3 h (group A) or 6 h (group B) in RPMI medium were presented
(see Table S3 in the supplemental material).

RESULTS

Differences in growth and quorum-sensing-like phenotype between <i>tup1</i>-Δ strains of serotypes A and D. <i>TUP1</i> deletion in
serotype D strains of <i>C. neoformans</i> resulted in growth retar-
dation, as reported for <i>tup1</i>-Δ strains of other fungal species,
but without any defects in yeast cell morphology or flocculation
(26). In order to investigate the effect of <i>TUP1</i> deletion in
serotype A strains, the <i>TUP1</i> gene was deleted and then com-
plemented in strain H99. Although the Tupal proteins from
serotype A (H99) and D (JEC21) strains share 94% amino acid
identity, deletion of the <i>TUP1</i> gene resulted in distinct pheno-
typic differences between the strains. While the H99 <i>tup1</i>-Δ
strain showed slight growth retardation compared to the wild-
type strain, it was less severe than what had been observed for
the serotype D <i>tup1</i>-Δ strain (see Fig. S2 in the supplemental
material) (26). The doubling times of the wild-type H99, <i>tup1</i>-Δ
(HL112), and complemented (HL132) strains were 2.23 h,
3.9 h, and 2.28 h, respectively, at 30°C and 4.08 h, 5.82 h, and
3.78 h, respectively, at 37°C in YES liquid medium. The slight
reduction in the growth rate of the <i>tup1</i>-Δ strain was also ob-
served on solid agar media such as YES and SD (see Fig. S2 in
the supplemental material, and data not shown). Therefore,
<i>TUP1</i> does not appear to influence cell proliferation in the
H99 strain as much as in serotype D strains.

One of the striking phenotypes observed previously with
serotype D <i>tup1</i>-Δ strains was the inoculum size threshold as a
prerequisite for normal growth, which mimics the quorum-
sensing phenomenon (27). A cell density of about 5 × 10<sup>5</sup>
was required for the strain HL14, the serotype D <i>tup1</i>-Δ strain, to
grow on SD medium (Fig. 1A, left panel). Tests to determine
whether cell density would similarly influence growth of the
<i>tup1</i>-Δ serotype A strain, however, did not show a density-
dependent growth phenotype in the H99 <i>tup1</i>-Δ strain (HL112)
(Fig. 1A, right panel).

To investigate whether the lack of density-dependent growth in
H99 is strain dependent or common among serotype A strains,
we deleted <i>TUP1</i> in several other serotype A strains. Three serotype A strains, CHC186, VNB163, and WM148, that
are genetically diverse based on their molecular genotype, such as
mini-and macrosatellite DNA, intergenic sequence, and ri-
bosomal DNA sequences as well as multilocus sequencing type
sequences of marker genes (6), were chosen to construct <i>tup1</i>-Δ
strains. <i>TUP1</i> was also deleted in WSC1156, the <i>MATa</i>
strain isogenic to H99. Consistent with the observations for H99,
deletion of <i>TUP1</i> in the backgrounds of all these serotype A
strains caused slight growth retardation but without the density-
dependent growth phenotype (data not shown).

In serotype D <i>tup1</i>-Δ strains, the inability to grow at low cell
densities could be rescued by supplementing the growth me-
dium with the culture filtrate from a high-cell-density <i>tup1</i>-Δ
culture. The active molecule in the culture supernatant respon-

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sible for the density-dependent growth phenotype was identified as an oligopeptide, quorum-sensing-like peptide 1 (QSP1) (27). QSP1 is an 11-amino-acid peptide that is processed from the CQS1 gene product (Fig. 1B). A CQS1 homolog, CNAG_03012.2, which encodes a hypothetical protein of 45 amino acids, is present in the H99 genome. The sequence of QSP1, the active peptide purified from serotype D tup1Δ culture filtrate, is in bold. Two amino acids in H99 Cqs1 which are different from those in JEC21 Cqs1 are underlined.

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**FIG. 1.** Deletion of TUP1 in H99 does not cause density-dependent growth. (A) Exponentially growing cultures (OD₆₀₀ of 0.5 to 1.0) were washed, resuspended in 0.9% NaCl, and plated on SD medium. Totals of 5 × 10⁶ and 10⁶ cells for LP1 (wild type) and its tup1Δ mutant (serotype D strains) and 5 × 10⁶ and 5 × 10⁵ cells for H99 (wild type) and its tup1Δ mutant (serotype A strains) were plated on SD medium and incubated for 2 days at 30°C. (B) Comparison of Cqs1 sequences in JEC21 (serotype D) and H99. The sequence of QSP1, the active peptide purified from serotype D tup1Δ culture filtrate, is in bold. Two amino acids in H99 Cqs1 which are different from those in JEC21 Cqs1 are underlined.

**FIG. 2.** TUP1 disruption affects mating and capsule formation. (A) Strains H99 (wild type; left panel) and HL112 (tup1Δ; right panel) were each mixed with KN99a on V-8 juice agar, incubated for 72 h, and observed for hyphal formation. (B) H99 (wild type [WT]), HL112 (tup1Δ), and HL132 (TUP1-complemented strain; tup1Δ + TUP1) were grown on RPMI agar medium at 37°C for 2 days and examined for capsule formation by microscopic examination of India ink slide preparations.

**NOTE:**

C. neoformans serotype D strains, is the regulation of sexual reproduction (26, 42, 46). We tested the effect of TUP1 deletion on mating in the serotype A strain H99. Mating of the wild-type H99 strain with the tester strain KN99a on V-8 juice agar produced extensive hyphae after 3 days of incubation (Fig. 2A, left panel). In a cross between HL112 (tup1Δ) and KN99a, however, the production of hyphae was reduced significantly (Fig. 2A, right panel). Similar observations of reduced mating have been reported for tup1Δ strains of serotype D (26). Therefore, the role of TUP1 in sexual reproduction appears to be conserved in both serotype A and D strains.

Although we did not see any effect of tup1Δ deletion on capsule formation in serotype D strains, tup1Δ strains in a serotype A background produced capsules that were significantly enlarged compared to those of wild-type strains. Figure 2B shows the markedly increased capsule size in HL112, the tup1Δ strain of H99, compared to HL132, the tup1Δ + TUP1 strain, and the wild-type strain H99. Deletion of TUP1 in other genetically unrelated serotype A strains also showed significant increases in capsule size (data not shown). These observations clearly associate the hypercapsular phenotype with the deletion of TUP1 in serotype A strains. The enlarged capsule formation in serotype A tup1Δ strains was consistent regardless of growth media at both 30°C and 37°C. Among all the media tested, RPMI induced the most pronounced difference in capsule size between wild-type and tup1Δ strains (Fig. 2B).

**TUP1 regulates expression of genes involved in capsule formation and iron/copper homeostasis in serotype A strain H99.**

Given the prominent hypercapsular phenotype of serotype A tup1Δ strains, it is possible that TUP1 might alter the expression levels of genes involved in capsule formation. Gene profile analysis was undertaken as a preliminary screen to identify the putative genes whose expression was affected by TUP1 dele-
tion. Microarray analysis was performed on the H99 and tup1Δ strains grown for 3 h and 6 h in RPMI liquid medium at 30°C. The expression levels of a few genes involved in capsule biosynthesis and iron/copper homeostasis were affected by deletion of TUP1 in H99 (see Table S3 in the supplemental material). To confirm the expression patterns, mRNA levels of several genes were examined by real-time RT-PCR. Quantitative PCR results showed that the transcriptional levels of three genes involved in capsule synthesis, CAP10, CAP64, and CAS35, were about threefold higher in the tup1Δ strain than in H99 (Fig. 3). Conversely, the expression levels of three genes with an annotated function involved in iron/copper homeostasis, CTR4, FRT1, and SIT2, were two- to ninefold lower in the tup1Δ strain (Fig. 3) (28). In addition, the expression of CIG1, which encodes a product believed to be an extracellular mannosprotein involved in the retention of iron at the cell surface and/or in the uptake of siderophore-bound iron (28), was downregulated by twofold in the tup1Δ strain. However, the expression of CFT2, an ortholog of the Saccharomyces cerevisiae high-affinity iron permease gene FTR1 in C. neoformans, was not affected by the deletion of TUP1 (17).

Since TUP1 affected the expression of genes involved in iron and copper uptake/homeostasis, growth of the tup1Δ strain was tested on both iron-chelated medium (LIM + EDDA) and iron-replete medium (LIM + Fe). Interestingly, growth of the tup1Δ strain was reduced on LIM + EDDA medium compared to that of the wild-type strain, and iron repletion (LIM + Fe) restored growth of the tup1Δ strain (Fig. 4A). Furthermore, on LIM medium treated with a chelator to remove copper (LIM + BCS + EDDA), the growth difference between the wild-type and tup1Δ strains was even more drastic (Fig. 4B, right panel). These results indicated that TUP1 regulates the utilization of iron and copper, which corroborates the expression data for several iron/copper homeostasis genes affected by the deletion of TUP1 in H99. Melanin production is one of the known virulence factors in C. neoformans, and iron/copper homeostasis can affect melanin production (14).
duction in the tup1Δ strains, examined on norepinephrine-containing medium, was observed to be notably reduced compared to that in the wild-type and complemented strains (Fig. 4C, left panel). Laccase is a key enzyme for melanin biosynthesis in C. neoformans. Since it requires four bound copper ions (43, 44) and copper is known to suppress the defect in melanin formation caused by mutation in genes involved in metal ion homeostasis (48, 49), the effect of Cu2+ on the melanin phenotype of the tup1Δ strain was studied. Melanin production was apparently restored in the tup1Δ strain by supplementing the growth medium with 10 μM CuSO4. These results suggest that the tup1Δ strain is defective in copper homeostasis affecting melanin production (Fig. 4C, right panel).

An insufficient iron concentration in the growth environment is known to induce large capsules in C. neoformans (15). Although the tup1Δ strain already showed an enlarged capsule in RPMI medium, it was interesting to determine if iron levels still influence the capsule size. The capsule size was measured after growing cells on RPMI, LIM, and LIM+Fe agar plates (Fig. 4D). In concordance with previous studies, strain H99 produced larger capsules in LIM (2.51 ± 0.71 μm; n = 33) than in RPMI (1.87 ± 0.39 μm; n = 25) and iron-replete medium (LIM+Fe) (0.64 ± 0.22 μm; n = 24) (Fig. 4D, upper panels). In the tup1Δ strain, cells grown on RPMI were already hypercapsulated compared to H99 cells, but the capsules became even larger when cultured on LIM. The capsule size was significantly reduced upon addition of Fe to LIM (RPMI, 5.34 μm ± 1.21 [n = 14]; LIM, 6.14 μm ± 2.98, [n = 26]; and LIM+Fe, 2.47 μm ± 0.62 [n = 49]) (Fig. 4D, middle panels). The TUP1-complemented strain (HL132) behaved similarly to the wild-type strain in all growth conditions (Fig. 4D, bottom panels). Thus, deletion of TUP1 results in the formation of an enlarged capsule under non-iron-limiting conditions, which can be altered further in tup1Δ cells by iron levels in the environment.

**TUP1 deletion affects cell wall integrity and susceptibility to fluconazole.** Cir1 is another cryptococcal transcriptional regulator involved in iron homeostasis. CIR1 (CNAG_04864) is important for capsule formation and negatively regulates laccase expression in H99 (18). In addition, it has been shown that cir1 mutants are sensitive to sodium dodecyl sulfate (SDS) and the azole drug fluconazole, suggesting that Cir1 is involved in cell wall integrity and membrane functions (18). Since capsule and melanin production were affected in the tup1Δ strain, the effect of TUP1 deletion relative to changes in CIR1 expression was examined. Quantitative RT-PCR results showed CIR1 expression to be mildly affected in the tup1Δ strain compared to H99, as the relative expression level was only 1.36-±0.05-fold higher in the tup1Δ strain. Furthermore, expression of iron permease genes, such as CFT1 and CFT2, which are regulated by CIR1, was not affected by the deletion of TUP1 according to our microarray data (see Table S3 in the supplemental material). These data suggested that TUP1 does not regulate iron homeostasis through the CIR1 regulatory circuit. However, growth of the tup1Δ strain was significantly hampered in the presence of 0.01% SDS, and the tup1Δ strain displayed increased sensitivity to fluconazole (Fig. 4E). These data suggested that TUP1 is also involved in cell wall integrity and membrane functions.

**TUP1 deletion reduces virulence.** Since deletion of TUP1 in H99 resulted in both positive and negative effects with respect to the three major C. neoformans virulence factors, which include growth at 37°C and formation of melanin and capsule, its effect on virulence was investigated. Groups of 10 mice were challenged with different yeast stains via tail vein injection. Figure 5A shows that all mice challenged with the wild-type or the complemented strain succumbed to infection by 9 days postinjection, while it took 20 days for all mice infected with the tup1Δ strain to succumb (P<0.001 compared to wild-type-infected mice), indicating that dele-
tion of TUP1 causes attenuation of virulence in C. neoformans.

To examine the pathobiological differences in mice infected with the wild-type or tup1Δ strain, the brain fungal burden and capsule size were determined at different stages of infection. Significant differences in the number of CFU were observed for the H99 and tup1Δ strains (5.93 × 10³ versus 5.3 × 10² per brain) as early as 2 days after injection (Fig. 5B). The number of CFU, however, increased exponentially and differed even more at 6 days after injection. These data suggest that TUP1 is important for growth in vivo, although the tup1Δ strain only showed a marginal reduction in growth at 37ºC in vitro. Another noteworthy observation was that fungal burden analyzed on the day of death in a mouse injected with the tup1Δ strain (1.25 × 10³ at day 13) was 80-fold lower than that in a mouse infected with H99 (8.35 × 10⁸ at day 9) (Fig. 5B). It is possible that the larger capsule size in the tup1Δ strain in vivo (Fig. 2A) might have contributed to such a difference. Surprisingly, the capsule size of the yeast cells in the brain smear from mice infected with tup1Δ strain was similar to that for mice infected with H99 (Fig. 5C). These findings clearly indicate that Tup1 plays an important role in the pathobiology of C. neoformans.

**DISCUSSION**

This study investigated the function of TUP1 in C. neoformans serotype A strains, including H99, and presents another example of serotype-specific difference in gene regulation. TUP1 plays a conserved role with respect to growth and mating but distinctly different roles in strains of serotypes A and D. Serotype A-specific phenotypes of tup1Δ strains include a lack of density-dependent growth, an enlarged capsule size, reduced melanin production, and a defect in iron/copper homeostasis.

The prominent capsule size in the tup1Δ strain under non-inducing conditions indicated the important role of TUP1 in capsule formation. Many environmental factors have been shown to influence the size of the capsule in C. neoformans. Low concentrations of glucose and iron, high concentrations of carbon dioxide, and the presence of serum components have shown to influence the size of the capsule in C. albicans (50). Whether C. neoformans and C. albicans fep1+ encodes a GATA transcription factor that represses the expression of iron uptake genes has been shown in Schizosaccharomyces pombe (50). S. pombe fep1+ encodes a GATA transcription factor that represses the expression of iron transport genes in response to elevated iron levels. Using yeast two-hybrid analysis, it has been shown that Tup1, a homolog of S. pombe, and Fep1 physically interact with each other (50). Whether C. neoformans Tup1 directly interacts with the proteins involved in iron homeostasis is yet to be determined.

The importance of copper homeostasis in C. neoformans is suggested by the copper dependency of two well-known virulence factors, the Cu/Zn superoxide dismutase (7) and laccase, a key enzyme in melanin synthesis (37, 43). Both enzymes require copper as a cofactor for their function. Deletion of C. neoformans LAC1, encoding the laccase, or mutation in the copper-binding site of the gene resulted in a significant reduction in virulence (37, 43). Copper also induces laccase transcription in wild-type cells and can restore laccase activity in vph1Δ mutants (48). In addition, the close relationship between copper and iron homeostasis has been reviewed (16).
For example, copper homeostasis also affects iron, since Fet3, the high-affinity iron transporter, requires the incorporation of four copper ions for the function (16). Thus, ineffective copper loading of Fet3 due to a defect in copper homeostasis can also lead to lower intercellular levels of iron, which affects capsule and melanin production. In fact, mutation in CCA2 (encoding a copper transporter) or ATX1 (encoding a copper chaperone) resulted in large capsules under iron-replete conditions and impaired growth under iron-limiting condition (40). Although our microarray data did not show significant changes in CCA2 and ATX1 gene expression (see Table S3 in the supplemental material), reduced expression of CTR4 (encoding copper transporter 4) in the tup1Δ strain and additive growth defects of the tup1Δ strain in iron/copper-chelated media lend additional support for the regulatory role of TUP1 in both iron and copper homeostasis.

Another intriguing result of our study is that Tup1 appears to function as both a repressor and an activator in C. neoformans. In contrast to the prevailing view of Tup1 as a global repressor, our results showed that many genes were also down-regulated in the absence of TUP1, suggesting that Tup1 functions as an activator for the expression of those genes. An analogy is seen with Hap1 in S. cerevisiae, which was originally identified as a heme-dependent transcriptional activator but was reported to function also as transcriptional repressor, depending on oxygen levels (11). Mammalian nuclear hormone receptors also are examples of factors that can act both positively and negatively through the recruitment of coactivators and corepressor complexes, respectively (45). Conversely, it is also possible that the downregulated genes in the tup1Δ strain are due to the indirect effect of Tup1. For instance, Tup1 could interact with a negative regulator, and inactivation of Tup1 could lead to the activation of a negative regulator, which in turn would cause the observed downregulation of genes in the tup1Δ strain. Additional experiments are required to identify the direct target(s) of Tup1 and possible interacting partners, if any, to understand the mechanism of Tup1 regulation in C. neoformans.

In C. albicans, disruption of TUP1 causes an inability to switch between yeast and filamentous forms and results in constitutive filamentous growth, which presumably is the reason why the tup1Δ strain is avirulent (2, 3). Previously, virulence studies could not be carried out properly with tup1Δ strains in a serotype D background because of their inability to grow at low cell densities, which hindered the precise determination of inoculum size based on CFU (26, 27). Here, we showed that deletion of TUP1 in H99 affected virulence. Since the TUP1 deletion displayed pleiotropic effects, it is likely that the reduced virulence of the tup1Δ strain resulted from the combination of these effects and is possibly related to iron/copper homeostasis. Given the global regulatory role of TUP1 in fungi and the manifestation of different phenotypes in serotype A and D tup1Δ strains, an in-depth analysis of TUP1 function would offer a valuable tool toward understanding the divergence of gene regulation in C. neoformans.

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