Diverse Hap43-Independent Functions of the Candida albicans CCAAT-Binding Complex

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The CCAAT motif is ubiquitous in promoters of eukaryotic genomes. The CCAAT-binding complex (CBC) is conserved across a wide range of organisms, specifically recognizes the CCAAT motif, and modulates transcription directly or in cooperation with other transcription factors. In Candida albicans, CBC is known to interact with the repressor Hap43 to negatively regulate iron utilization genes in response to iron deprivation. However, the extent of additional functions of CBC is unclear. In this study, we explored new roles of CBC in C. albicans and found that CBC pleiotropically regulates many virulence traits in vitro, including negative control of genes responsible for ribosome biogenesis and translation and positive regulation of low-nitrogen-induced filamentation. In addition, C. albicans CBC is involved in utilization of host proteins as nitrogen sources and in repression of cellular flocculation and adhesin gene expression. Moreover, our epistasis analyses suggest that CBC acts as a downstream effector of Rhb1-TOR signaling and controls low-nitrogen-induced filamentation via the Mep2-Ras1-protein kinase A (PKA)/mitogen-activated protein kinase (MAPK) pathway. Importantly, the phenotypes identified here are all independent of Hap43. Finally, deletion of genes encoding CBC components slightly attenuated C. albicans virulence in both zebrafish and murine models of infection. Our results thus highlight new roles of C. albicans CBC in regulating multiple virulence traits in response to environmental perturbations and, finally, suggest potential targets for antifungal therapies as well as extending our understanding of the pathogenesis of other fungal pathogens.

The cis-acting CCAAT motif is one of the most ubiquitous sequences in the promoters of eukaryotic genomes (1–3) and is present in at least 30% of eukaryotic genes (4). The CCAAT-binding complex (CBC) is a highly conserved heterometric protein complex that is present in fungi, plants, and mammals and specifically recognizes the CCAAT motif (1). CBC in mammals and plants is called NY-Y (for nuclear factor Y) and is composed of three subunits: NY-YA, NY-YB, and NY-YC. NY-Y is sufficient for both DNA binding and transcriptional regulation (1). Precise homologs of NY-Y components have been designated in fungi, including Hap2/3/5 in Saccharomyces cerevisiae (5–8), Hap2 and Hap3 in Kluyveromyces lactis (9, 10), Php2/3/5 in Schizosaccharomyces pombe (11, 12), HapB/C/E in Aspergillus species (reviewed in reference 13), Hap2/3/5 in Cryptococcus neoformans (14), and Hap2/3/5 in Candida albicans (15–18). The NY-YB homologs (Hap3/Php3/HapC) and NY-YC homologs (Hap5/Php5/HapE) contain a highly conserved histone-like motif which is mainly responsible for nonspecific DNA binding as its function in histone H2A-H2B dimers (1). Moreover, the NY-YA homologs (Hap2/Php2/HapB) contain sequences that help to stabilize the heterotrimeric CBC and also contribute to DNA binding in a CCAAT-specific manner (1, 19). In addition, Aspergillus HapB carries nuclear localization signals that facilitate the import of whole CBC into the nucleus (20, 21).

In contrast to the case of NY-Y, the function of the S. cerevisiae Hap2/3/5 complex in transcriptional activation depends on a fourth subunit, Hap4 (22). Hap4 interacts with the Hap2/3/5 complex via a fungus-specific Hap4 recruitment domain within Hap5 (11) and positively regulates respiration (23). HAP4 transcription is induced by nonfermentable carbon sources (22). Recently, Hap4 homologs have been identified in many other fungi (14, 24–26), including C. albicans (16, 17). However, the Aspergillus nidulans homolog HapX, the S. pombe homolog Php4, the C. neoformans homolog HapX, and C. albicans Hap43 were found to act as transcriptional repressors instead of activators and to be involved in regulating iron homeostasis by negatively regulating expression of iron-consuming genes under low-iron conditions. Moreover, genomewide studies also revealed that C. albicans Hap43 and C. neoformans HapX play both positive and negative roles in modulating transcriptional responses to iron deprivation (14, 16). Notably, Hap4 homologs in pathogenic fungi, including Aspergillus fumigatus HapX, C. neoformans HapX, and C. albicans Hap43, are required for virulence (14, 16, 17, 27).

The functions of the NY-Y complex are quite diverse. This complex has general regulatory activities in gene expression and controls different sets of genes in different organisms or cell types. For instance, NY-Y in mammals can regulate the cell cycle, apoptosis, and cell self-renewal (1, 2), especially in hematopoietic stem cells (28). In hepatocytes, inactivation of NY-Y leads to hepatocellular degeneration, lipid deposition, and endoplasmic reticulum stress (29). NY-Y can also cooperate with the tumor suppressor p53 to determine cell fate (reviewed in reference 30). Furthermore, NY-F regulates gene expression in lymphocytes and astrocytes (31). Remarkably, plant NY-Y participates in diverse processes, such as control of drought stress, endoplasmic reticulum stress, and flowering time, as well as in development of the em-

Received 18 January 2013. Accepted 26 March 2013
Published ahead of print 29 March 2013
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Supplemental material for this article may be found at http://dx.doi.org/10.1128/EC.00014-13.
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doi:10.1128/EC.00014-13
bryozoans, nodule, and root (reviewed in reference 32). In fungal eukaryotes, most studies on CBC have focused on the cooperation of CBC with Hap43/HapX in regulating iron homeostasis and expression of iron-responsive genes. However, a recent study demonstrated that A. nidulans CBC also serves as a redox sensor that coordinates with cellular oxidative responses (33), suggesting that fungal CBC may possess functions other than iron-responsive and HapX-dependent functions.

In C. albicans, CBC was first shown to negatively regulate components of the mitochondrial electron transport chain in response to various carbon sources (15). Later, accumulating evidence highlighted both positive and negative roles for Hap43/CBC in regulating iron homeostasis (16–18, 34, 35). In addition, a genomewide phenotypic study demonstrated that deletion of C. albicans CBC (HAP5 [orf19.1973], HAP31 [orf19.517], and HAP2 [orf19.1228]) leads to increased resistance to the Tor1 kinase inhibitor rapamycin (36). Interestingly, the rapamycin-resistant phenotype is not observed in the hap34Δ mutant (36). Moreover, a regulatory role for Hap32 (also known as Hap3 [orf19.4647]) was predicted in a systemic interspecies signaling network (37) and verified in an in vitro infection model (38). Taken together, these studies imply that C. albicans CBC may potentially function in a Hap43-independent manner involving the target of rapamycin (TOR) signaling pathway, contributing to virulence.

In this study, we assessed the Hap43-independent functions of C. albicans CBC and found that CBC contributes to the regulation of non-iron-responsive virulence traits. We uncovered novel roles for CBC in activating low-nitrogen-induced filamentation and nitrogen acquisition from host proteins. In addition, our results show that CBC acts as a negative regulator of adhesin gene expression when environmental conditions are unfavorable for filamentation. Moreover, using DNA microarray and epistasis analyses, we demonstrated that CBC acts as an important effector downstream of Rhb1-TOR signaling and also as a link between the TOR and Mep2-Ras1-protein kinase A (PKA)/mitogen-activated protein kinase (MAPK) pathways. Finally, deletions of individual CBC components attenuated C. albicans virulence in both zebrafish and murine models of infection. In summary, these findings describe new roles for CBC and correlate CBC function with iron-responsive and HapX-dependent functions.

DNA microarray analysis. For microarray analysis, cells were grown in YPD medium at 30°C overnight and subsequently diluted in fresh YPD medium to an optical density of 0.60 (OD600) of 0.5. After 4.5 h of incubation, cultures were treated with 0.2 μg/ml rapamycin for 0.5 h with shaking at 30°C. Total RNA was extracted using the phenol-chloroform method, and genomic DNA was removed using Turbo DNase (Ambion) (17). Purified RNA was quantified by measuring the OD260, using an ND-1000 spectrophotometer (Nanodrop Technology), and the RNA quality was checked using a Bioanalyzer 2100 instrument (Agilent Technologies) with an RNA 6000 Nano LabChip kit (Agilent Technologies). For cDNA synthesis, 0.2 μg total RNA was amplified using a Low Input Quick-Amp labeling kit (Agilent Technologies) and labeled with Cy3 (CyDye: Agilent Technologies). Cy3-labeled cRNA (0.6 μg) was fragmented to an average size of ~50 to 100 nucleotides by incubating in fragmentation buffer at 60°C for 30 min. Fragmented labeled cRNAs were subsequently pooled and hybridized to a custom C. albicans 21 Oligo 8 × 15K microarray (Agilent Technologies) at 65°C for 17 h. After washing and drying with nitrogen gun blowing, microarray slides were scanned at 535 nm, using an Agilent microarray scanner. Scanned images were analyzed with Feature extraction 10.5.1.1 software (Agilent Technologies). For design of the gene probes, sequences of 6,205 C. albicans transcripts of strain SC5314 (assembly 21) were extracted from the Candida Genome Database (http://www.candidagenome.org/) and uploaded to Agilent eArray. The probes were analyzed by base composition methodology. Duplicate sequences were removed, resulting in the generation of 6,202 gene probes. The 6,202 specific probes were printed in situ syn-
thesis in duplicate on each array, with an 8-by-15,000 format (Agilent Technologies).

For microarray data analysis, GeneSpring GX 11.5 software (Agilent Technologies) was used for normalization and expression analysis. Probability scores were calculated with a default t test of the ratio of median values (hap5Δ mutant/wild type) from four independent replicates. Gene expression differences with P values of <0.05 were considered significantly different. Genes whose expression was significantly different in the hap5Δ mutant and had an expression level ≥2-fold higher or lower than that of the wild type were selected for gene ontology (GO) analysis.

Real-time quantitative PCR (qPCR). Cells were grown in YPD or SC medium at 30°C overnight and subsequently diluted in fresh medium to an OD600 of 0.5. After 5 h (for iron-containing media), 7 h (for nitrogen-containing media), or 22.5 h (for YCB/BSA medium) of incubation, cells were harvested by centrifugation. Cells treated with rapamycin were preincubated in fresh high-iron or low-iron medium to an OD600 of 0.5. After 5 h of incubation, cells were harvested and serially diluted to the desired concentration.

Promoter analysis. Promoter activities were evaluated by measuring the level of β-galactosidase (β-Gal) reporter (43), using liquid β-Gal assays and X-Gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside) overlay assays as described previously (17). For liquid assays, cells were grown overnight in YPD medium were diluted in sterile fresh high-iron or low-iron medium to an OD600 of 1.0. A total of 5 µl of each cell suspension was spotted onto high-nitrogen and low-nitrogen agar plates. The cells were incubated at 37°C for 8 days, and colony spots were photographed. For liquid assays, cells grown overnight in SC medium were harvested and serially diluted to the desired concentration. For overlay assays, cells grown overnight in YPD medium were diluted in sterile dH2O to a density of 5 OD600 units/ml, and 5 µl of each cell suspension were spotted onto high-nitrogen and low-nitrogen agar plates. The cells were incubated at 30°C overnight, lysed, and used for the X-Gal–agarose overlay. The overlay plates were then incubated at 30°C until a blue color developed.

Phenotypic assays. To assay for filamentation, cells were grown in SC medium at 30°C overnight and subsequently diluted in sterile dH2O to an OD600 of 1.0. A total of 5 µl of each cell suspension was spotted onto high-nitrogen and low-nitrogen agar plates. Neutral nitrogen medium was adjusted with 50 mM HEPES buffer (pH 7.0). The plates were incubated at 37°C for 8 days, and colony spots were photographed. For evaluation of cell growth by spot assays, iron-starved cells or cells grown overnight in SC medium were harvested and serially diluted to the desired cell densities with sterile dH2O. Each diluent was spotted onto agar plates (5 µl/spot) and incubated at 30°C for 1 day or longer, as indicated. For flocculation assays, cells from a single colony were grown in YPD medium or other medium, as indicated, overnight in a 24-well plate or in 50-ml tubes with shaking at 180 rpm at 30°C. Aliquots of flocculated cells were observed at a magnification of ×40 with a Zeiss upright microscope (Zeiss Imager A1). For sedimentation assays, cells cultured overnight were transferred to a plastic flow tube by use of a 25-ml pipette and photographed immediately after transfer. The sedimentation assays were performed at room temperature and monitored for at least 28 h. To assay for collocu-

lation, colonies from two different strains were inoculated into the same culture and incubated as described above. To observe the fluorescent flocs, flocculated cells were suspended by slow orbital shaking, and aliquots of flocculated cells were observed at a magnification of ×40 with a Zeiss upright microscope equipped for epifluorescence imaging.

Virulence assay. Peritoneal infections of zebrafish were performed as described previously (46), with some modifications. Briefly, fresh single colonies were inoculated into 10 ml SC medium and incubated at 30°C for 24 h with shaking at 180 rpm. Cells were harvested by centrifugation, washed with sterile phosphate-buffered saline (PBS), and resuspended in sterile PBS at a cell density of 1 × 1010 CFU/ml. Zebrafish were anesthetized by immersion in water with 170 mg/liter of tricaine (Sigma). C. albicans cell suspensions (10 µl) were injected into the peritoneal cavity of anesthetized zebrafish by use of a 26.5-gauge syringe (701N syringe; Hamilton), and the fish were immediately allowed to recover in fresh water. Zebrafish injected with different C. albicans strains were kept in independent 5-liter tanks in which the water was changed daily. All tanks were housed at 28.5°C, with a cycle alternating between 14 h of light and 10 h of darkness. The fish were monitored every 1 to 2 h for 5 days.

For the assay of C. albicans infection in mice, female BALB/c mice (7 weeks old) were obtained from BioLasco Taiwan Co., Ltd., and housed (five mice per cage) for 1 week before experiments. C. albicans cells grown in SC medium overnight were subcultivated into SC medium at 30°C with shaking at 180 rpm for 4 h to reach the early log phase. Cells were harvested, washed with PBS, and resuspended in PBS at a density of 1 × 107 CFU/ml. The cell suspension (1 × 107 cells) was injected into the lateral tail vein of each mouse. The infected mice were monitored twice per day for 3 weeks. The animal studies were approved by the Institutional Animal Care and Use Committees of the National Tsing Hua University and Animal Technology Institute Taiwan, Taiwan. The log rank test was used to assess the differences in survival between groups of fish or mice. A P value of <0.05 was considered statistically significant.

Microarray data accession number. The microarray data were submitted to the Gene Expression Omnibus website (http://www.ncbi.nlm.nih.gov/geo/) under accession number GSE41266.

RESULTS

Regulatory roles for CBC in gene expression and cell growth in different environments. In order to explore new functions of CBC, we constructed various mutants lacking different components of CBC. As indicated above, CBC functions with Hap43 to regulate iron homeostasis in C. albicans. Therefore, to ensure successful construction, we first assessed the roles of these newly constructed CBC mutants and the hap43Δ mutant in iron-dependent cell growth and iron-responsive gene regulation. Cells were grown on YPD agar plates with a low (200 µM BPS) or high (no BPS) concentration of free iron. Our results were consistent with those of Homann et al. (36), which indicated that hap43Δ, hap5Δ, and hap2Δ mutants were much more sensitive to iron chelation than the wild type. Another study reported that HAP32 contributes to cell growth under low-iron conditions (16); however, we found that HAP32 and HAP31 appear to have redundant functions in growth under low-iron conditions (see Fig. S1A in the supplemental material). Moreover, when iron was depleted, misexpression of iron utilization genes such as CCP1, ACO1, and YAH1 was observed in hap43Δ, hap5Δ, and hap2Δ mutants (16, 17). Consistent with the pattern shown in the growth assay (see Fig. S1A), hap43Δ, hap5Δ, and hap2Δ mutants showed a similar gene derepression to that in previous studies (16, 17). In addition, complementation of the hap5Δ mutant verified that the engineered mutation was the cause of the mutant phenotype (see Fig. S5). Interestingly, single deletion of HAP31 or HAP32 did not lead to derepression of iron utilization genes in this study (see Fig. S1B). Together, these re-
results validated that the newly constructed CBC mutants, as well as the \(h_{ap43}\Delta\) mutant, affect the iron homeostasis of \(C.\ albicans\).

In addition to the role of CBC in iron homeostasis, other functions of CBC were further studied. Rapamycin is an immunosuppressive drug that can form a complex with the conserved FKBP12 protein (rapamycin-binding protein) to inhibit the activity of TOR kinase (47, 48). In \(S.\ cerevisiae\), TOR inhibition by rapamycin triggers the expression of nitrogen catabolite repressed (NCR) genes and blocks cellular growth by inhibiting ribosome biogenesis and translation and inducing autophagy (48). Rapamycin has been used to induce responses mimicking those of cells undergoing nitrogen starvation in \(S.\ cerevisiae\) and \(C.\ albicans\) (40, 44, 49, 50). The wild type and the \(h_{ap43}\Delta\) mutant were sensitive to rapamycin (Fig. 1A). Therefore, the JRB12 (TOR1/TOR1-1) strain, containing a dominant rapamycin-resistant TOR\(^{S1984I}\) allele (51), was used as a strong rapamycin-resistant control (Fig. 1A). Except for the \(h_{ap32}\Delta\) strain, rapamycin did not inhibit the growth of other CBC mutants. This result is consistent with the results of a previous study (36). Moreover, we compared the expression of NCR genes in CBC mutants in response to rapamycin (Fig. 1B). As controls, MEP2 and SAP2 were induced by rapamycin in both the wild-type and \(h_{ap43}\Delta\) strains. MEP2 was not induced by rapamycin in the rapamycin-resistant CBC mutants, including the \(h_{ap5}\Delta, h_{ap31}\Delta, h_{ap32}\Delta h_{ap31}\Delta,\) and \(h_{ap2}\Delta\) strains. Interestingly, rapamycin induction of \(SAP2\) expression was decreased in the \(h_{ap5}\Delta, h_{ap32}\Delta h_{ap31}\Delta,\) and \(h_{ap2}\Delta\) mutants but not in the \(h_{ap31}\Delta\) strain. Moreover, expression of the NCR gene \(GAP2\), which lacks a CCAAT motif in the promoter, was not affected by CBC deletion. Finally, the iron-responsive gene \(CCP1\) was used as a negative control for rapamycin treatment, and as expected, its expression was not significantly affected by rapamycin or deletion of CBC components. Furthermore, one of the CCAAT motifs was required for MEP2 expression in response to low-nitrogen conditions (see Fig. S2 in the supplemental material). Therefore, we concluded that CBC has functions other than regulating iron homeostasis and is responsible for both growth arrest and induction of gene expression in response to TOR inhibition.

**Differential expression of the two Hap3 paralogs, Hap31 and Hap32.** As shown in Fig. 1A and B, only deletion of HAP31 was sufficient to abolish the function of CBC in response to rapamycin. However, deletion of both HAP32 and HAP31 was required to attenuate the low-iron responses of cells (see Fig. S1 in the supplemental material). We thus speculated that CBC components may assemble into different complexes, and this hypothesis was supported by the results of pairwise interactions between CBC components from two-hybrid analyses (see Fig. S3A). Our data demonstrate that Hap32 and Hap31 can each interact with Hap5 or Hap2. However, Hap32 and Hap31 do not bind with each other. Furthermore, gene expression analysis indicated that the HAP31 and HAP32 genes were differentially expressed in response to various conditions. HAP31 was expressed at the highest levels under high-iron conditions (YPD without BPS), even in the presence of rapamycin (see Fig. S3B and C in the supplemental material). Because rapamycin treatment mimics a nitrogen depletion stimulus (49), we also assayed HAP31 expression during nitrogen depletion conditions. We found that HAP31 was upregulated under low-nitrogen conditions, including in low-ammonium and BSA-only media (see Fig. S3D). However, HAP32 expression was induced by low iron and slightly repressed in response to rapamycin (see Fig. S3B and C). Moreover, HAP32 expression remained constant under both high- and low-nitrogen conditions (see Fig. S3D). For the combination of two-hybrid and gene expression analyses, the results suggest that CBC in \(C.\ albicans\) can be composed of either Hap5, Hap32, and Hap2 or Hap5, Hap31, and Hap2.

To further test the importance of differential expression of HAP32 and HAP31 in CBC functions, we monitored the complementary activity of expression following promoter swapping of HAP32 or HAP31 in the strain with the \(h_{ap32}\Delta h_{ap31}\Delta\) background. A relatively low level of HAP31 expression driven by the HAP32 promoter converted the rapamycin-resistant \(h_{ap32}\Delta\)
FIG 2 Excess Hap32 or low levels of Hap31 can complement the rapamycin resistance of the hap3Δ mutant. Overnight cultures were serially diluted and spotted onto YPD agar plates in the absence or presence of 10 ng/ml rapamycin. The plates were incubated at 30°C for 2 days. Two independent clones of promoter-swapped strains were used. Expression of HAP32 by the high-iron-induced HAP31 promoter in YPD-based medium (high iron) failed to complement the phenotype of increased rapamycin resistance in the hap3Δ hap31Δ mutant. Basal expression of HAP31 by the low-iron-induced HAP32 promoter in YPD-based medium decreased the resistance to rapamycin of the hap3Δ hap31Δ mutant. Moreover, overexpression of HAP32 by the ADH1 promoter in the hap31Δ mutant decreased the cell resistance to rapamycin and restored it to the level of the wild type.

hap3Δ double mutant to a rapamycin-sensitive strain (Fig. 2, upper panels). However, the higher expression of HAP32 driven by the HAP31 promoter did not complement the increased rapamycin resistance in the hap3Δ hap31Δ strain (Fig. 2, upper panels). Because the expression of HAP32 driven by the HAP31 promoter was insufficient to restore rapamycin sensitivity, we strongly increased the level of HAP32 by ectopically expressing HAP32 with the constitutive ADH1 promoter in the hap31Δ

FIG 3 Comparisons between the hap5 null mutant and the wild type in their responses regarding rapamycin-mediated gene expression. (A) Comparison of gene expression by DNA microarray analysis. Overnight cultures of the hap5Δ mutant and the wild type were subcultured in YPD at 30°C for 4.5 h, followed by incubation for 0.5 h in the presence of 0.2 μg/ml rapamycin. RNA transcripts were analyzed with DNA microarrays. Data shown are for genes with fold changes of ≥2 and P values of <0.05. The fold change of each gene was calculated as the hap5Δ expression level divided by the wild-type expression level. We identified 608 differentially expressed genes that are functionally classified according to gene ontology (www.candidagenome.org/cgi-bin/GO/goTermMapper) and manual curations. A list of the genes grouped according to GO terms is shown in Table S1 in the supplemental material. (B) Among the 608 differentially expressed genes, many are classified as Hap43-regulated genes (see Table S1) according to published microarray and ChIP-chip data (16, 34). (C) Verification of gene expression by qPCR. MEP2 and HAP32 expression was assayed in cells grown in high- and low-nitrogen media. SAP2 expression was assayed in cells grown in low- or high-nitrogen medium and YCB/BSA medium. Cells for RNA extraction were grown in the indicated media at 30°C for 7 h for low- or high-nitrogen medium and 22.5 h for YCB/BSA medium. Expression levels are displayed as means ± SD for at least two independent experiments.

Role of CBC in rapamycin-responsive gene regulation. To further understand the possible connection between TOR signaling and CBC, we compared the TOR-inhibited transcriptional profiles between a CBC mutant and the wild type by using DNA microarray analysis. Because a loss of HAP5 was sufficient to abolish CBC, the hap5Δ mutant was used as a loss-of-function strain for CBC. We identified 608 genes that were differentially expressed in the hap5Δ mutant compared to the wild type in response to rapamycin treatment (see Table S1 in the supplemental material). Among them, 343 genes were upregulated in the hap5Δ mutant, and more than 30% of these genes have functions involved in ribosome biogenesis, transcription/RNA processing, and translation (Fig. 3A). Strikingly, HAP3 (also known as HAP32) was one of the genes that were upregulated in the hap5Δ mutant in response to rapamycin. Moreover, 265 genes were downregulated in the hap5Δ mutant, including 39 genes responsible for nitrogen utilization and 35 genes related to transport of other molecules/nutrients. These genes constituted the largest proportion (~12%) of annotatable downregulated genes (Fig. 3A). As expected, the MEP2 and SAP2 genes were included in the list of downregulated genes. Taken together, these results were consistent with the results of the rapamycin growth assay (Fig. 1A) and suggested that the translational machinery and NCR gene regulation in the hap5Δ mutant are not affected by TOR inhibition.

Interestingly, only 25% of these differentially expressed genes
have been reported to be regulated by Hap43 (Fig. 3B), according to comparisons with previous DNA microarray data and chromatin immunoprecipitation-DNA chip (ChIP-chip) data (16, 34). This finding further suggests that CBC can function in a Hap43-independent manner, especially under conditions other than those with low iron.

Because rapamycin treatment of cells can induce responses similar to those in cells grown under nitrogen-depleted conditions and because a large percentage of genes involved in nitrogen utilization and metabolism were downregulated in the hap5Δ mutant with rapamycin treatment (see Table S1 in the supplemental material), we further examined the expression patterns of MEP2, HAP32, and SAP2 in hap5Δ cells under various nitrogen conditions (Fig. 3C). MEP2 was expressed at a lower level in the hap5Δ mutant than in the wild type under low-nitrogen conditions. HAP32 expression in the wild type remained constant under both nitrogen conditions. However, loss of HAP5 strongly increased HAP32 expression under low-nitrogen conditions. Moreover, expression of the major secreted aspartyl proteinase gene, SAP2, was diminished compared to that in the wild type when cells were grown in medium using BSA as the sole nitrogen source. Accordingly, our results highlighted the importance of CBC in regulating cellular responses to nitrogen depletion.

Positive role for CBC in regulating nitrogen utilization. Given that nitrogen utilization genes were downregulated in CBC mutants in response to rapamycin treatment, low-nitrogen conditions, or the presence of BSA in the medium, we evaluated the role of CBC in nitrogen utilization by growing cells under nitrogen-starved conditions. Cell growth in YCB/BSA medium is a short, our data demonstrate that CBC positively regulates nitrogen utilization and supports cell growth.

Regulation of low-nitrogen-induced filamentation by CBC. C. albicans Mep2 acts as an ammonium permease and a signal transducer involved in low-nitrogen-induced filamentation (52, 53). Because CBC can regulate MEP2 expression (Fig. 1B and 3C), we tested the possibility that CBC may have a role in low-nitrogen-induced filamentation by regulating Mep2. CBC mutants were spotted onto high-nitrogen and low-nitrogen agar plates and incubated at 37°C to induce filamentation (Fig. 5A). The CBC subunits Hap5, Hap31, and Hap2, but not Hap32, were found to be essential for low-nitrogen-induced filamentation, and this phenotype was Hap43 independent. This finding is consistent with the observation that HAP31 was upregulated under low-nitrogen conditions, in contrast to HAP32 (see Fig. S3D in the supplemental material). Moreover, our previous study reported that low-nitrogen-induced filamentation in C. albicans is regulated by the Rhb1-TOR pathway via its control of MEP2 (44). To determine whether CBC participates in this process, we constructed a hap5Δ rhb1Δ double mutant. This mutant is more resistant to rapamycin than the rhb1Δ mutant but more sensitive than the wild type, indicating that deletion of HAP5 can partially restore the phenotype of rapamycin hypersensitivity in the rhb1Δ mutant (Fig. 5B). Furthermore, the hap5Δ rhb1Δ double mutant phenocopied the hap5Δ single mutant in filamentation under low-nitrogen conditions (Fig. 5C). Consequently, epistasis analysis suggested that CBC acts downstream of the Rhb1-TOR pathway in C. albicans.

To further evaluate the role of Mep2 as a signal transducer in CBC-mediated filamentation, hap5Δ MEP2ΔC440 and hap5Δ RAS1G13V mutants were generated. As shown in Fig. 5D, native expression of the constitutively active MEP2ΔC440 allele (52) partially complemented the defect in low-nitrogen-induced filamentation in the HAP5 deletion mutant. Similarly, expression of the constitutively active RAS1G13V allele (52) in the hap5Δ mutant completely complemented the defect in low-nitrogen-induced filamentation (Fig. 5E). Taken together, these findings suggest that CBC may act as one of the components that connect Rhb1-
FIG 5 CBC is required for low-nitrogen-induced filamentation mediated by Mep2 and functions downstream of Rhb1-TOR. (A) Wild-type, hap5Δ, and CBC mutant cells were spotted onto high- and low-nitrogen agar plates and incubated at 37°C for 8 days. Nitrogen media at pH 7.0 were buffered with 50 mM HEPES. (B and C) Epistasis analysis to study the genetic relationship between Hap5 and Rhb1, using the hap5Δ rhb1Δ mutant. Results from two independent clones of the hap5Δ rhb1Δ mutant are shown. Deletion of HAP5 on the rhb1Δ background increased the rapamycin resistance and completely abolished the hyperfilamentation phenotype resulting from RHB1 deletion. (D and E) Epistasis analysis to study the genetic relationship between Hap5 and Mep2 or Ras1, using the hap5Δ MEP2Δ440 and hap5Δ RAS1Δ135 mutans, respectively. Results from two independent clones of the hap5Δ MEP2Δ440 mutant are shown. Native expression of MEP2Δ440 partially restored the filamentation defect in the hap5Δ mutant, whereas RAS1Δ135 overexpression fully recovered filamentation in the hap5Δ mutant.
TOR signaling with the Mep2-Ras1-PKA/MAPK pathway for controlling low-nitrogen-mediated filamentation.

Negative regulation by CBC of flocculation under conditions unfavorable for filamentation. Considering that the TOR pathway positively regulates cell growth and proliferation when nutrients are sufficient (48), we assessed the contribution of CBC under nutrient-rich conditions. Interestingly, after overnight growth in YPD medium, cells of the CBC mutants, including the hap5Δ, hap31Δ, hap32Δ hap31Δ, and hap2Δ mutants, formed large flocs, whereas cells of the wild-type, hap43Δ, and hap32Δ strains were generally dispersed (Fig. 6A). Upon closer inspection, the flocs from the hap5Δ, hap31Δ, hap32Δ hap31Δ, and hap2Δ mutants were composed of extensively aggregated pseudohyphae and yeast-like cells, while the wild-type, hap43Δ, and hap32Δ strains were entirely dispersed yeast-like cells (Fig. 6B). Moreover, the hap5Δ, hap31Δ hap31Δ, and hap2Δ mutants formed large flocs and sedimmented rapidly to the bottom of the test tubes, in contrast to wild-type cells and the hap43Δ and hap32Δ mutants, which did not form flocs in overnight cultures and sedimmented slowly (Fig. 6C, top panels). Some floculated cells even adhered to the side walls of the plastic test tubes (see samples at the 28th hour). Interestingly, the hap31Δ mutant displayed intermediate flocculation, sedimentation, and substrate adherence (Fig. 6C, top panels). Remarkably, acidic pH abolished the formation of large flocs and decreased cell adherence to the side walls of test tubes (Fig. 6C, middle panels). Even in an acidic environment, however, the aggregated hap5Δ, hap31Δ, hap32Δ hap31Δ, and hap2Δ cells still sediment faster than the wild-type, hap43Δ, and hap32Δ cells. Furthermore, removal of glucose from the rich medium reduced cell growth and completely diminished the difference in flocculation among all strains tested (Fig. 6C, bottom panels).

Because deletion of CBC components causes extensive cellular aggregation, we assessed the cell-cell interactions between mutant and wild-type cells. Wild-type cells were manipulated to constitutively express green fluorescent protein (GFP) and were coincubated with hap5Δ cells in YPD medium (Fig. 6D, top panel). We found that cocultivated cells still formed flocs after incubation overnight at 30°C, and microscopic examination showed that the fluorescent wild-type cells aggregated with the nonfluorescent hap5Δ cells, forming multilayered clumps (Fig. 6D, bottom panels). As a control, dispersed yeast-like cells existed when only wild-type cells were present. These data consequently reinforced the possibility that cellular aggregation of CBC mutants results from alterations in cell surface molecules.

Interestingly, the TOR pathway in C. albicans is responsible for repressing cell-cell adhesion and expression of the cell surface adhesin genes HWP1, ECE1, ALS1, and ALS3 (50). TOR inhibition by rapamycin induces rapid flocculation of wild-type cells in Spider medium at 37°C (50). Considering these studies, we monitored the expression of adhesin genes in CBC mutants (Fig. 6E). The expression of adhesin genes was derepressed in cells of the hap5Δ, hap32Δ hap31Δ, and hap2Δ mutants compared to the wild type. Interestingly, expression of adhesin genes was not affected by deletion of HAP31, suggesting that increased levels of adhesins on the cell surface may not be the only cause of flocculation in the CBC mutants.

In summary, our results show that CBC also has a vital role in maintaining a low level of surface adhesin expression and the dispersed cell type under nutrient-rich conditions (unfavorable for forming filaments).

**Contribution of CBC to C. albicans virulence.** We previously showed that deletion of HAP43 diminishes the virulence of C. albicans in a murine model of systemic candidiasis (17). Because Hap43 is a partner of CBC (16, 17), we further evaluated the effects of CBC deletion on C. albicans virulence by using a zebrafish model of peritoneal infection (46). Zebrafish injected with the hap5Δ, hap32Δ hap31Δ, hap2Δ, and hap43Δ mutants lived longer than those injected with wild-type or reconstituted strains (Fig. 7). Single deletion of either HAP31 or HAP32 had no significant effect on virulence. Furthermore, the results of this fish-killing assay were verified and confirmed with a mouse model of disseminated infection (see Fig. S4 in the supplemental material). Taken together, our data suggested that CBC plays a contributory role in C. albicans virulence.

**DISCUSSION**

CBC is conserved in eukaryotes and is responsible for diverse functions. Using a combination of mutagenesis, gene expression profiling, and epistasis analysis, we examined the contributions of CBC to C. albicans virulence traits in vitro. Our findings broaden the understanding of C. albicans CBC functions (Fig. 8B), whose previously described roles were only in respiration, carbon metabolism, and iron homeostasis. Our studies show that CBC is a positive regulator of low-nitrogen-mediated responses and a negative regulator of expression of adhesin-like genes. Moreover, the analysis comparing Hap31 and Hap32 indicates that CBC comprising Hap5, Hap31, and Hap2 plays dominant roles under both nutrient-rich and low-nitrogen conditions, whereas the Hap5-Hap32-Hap2 complex is important only under iron-deficient conditions. Interestingly, the Rhb1-TOR signaling pathway appears to regulate CBC under both nutrient-rich and low-nitrogen conditions. However, the upstream signaling pathway that controls CBC-mediated iron responses still remains unidentified.

**CBC is a downstream effector of TOR signaling under iron-independent conditions.** Enhanced rapamycin resistance in CBC mutants has been mentioned previously (36), but no further experiments have been conducted to show the link between CBC and the TOR signaling cascade. C. albicans Rhb1 is a small G protein that acts upstream of TOR and is responsible for activation of TOR (44). Therefore, deletion of RHB1 somehow mimics the effects of TOR inhibition by rapamycin. Our findings demonstrate that deletion of HAP5 completely abolished or attenuated the phenotypes resulting from RHB1 deletion, indicating that CBC is epistatic to Rhb1-TOR (Fig. 5B and C). These phenotypes have not been shown to be involved in cellular responses to iron availability, thus suggesting that the signaling flow from TOR to CBC differs from the unidentified iron-mediated signaling cascade.

A simple model for the various functions of CBC is proposed in Fig. 8A. In this model, CBC acts as a key effector downstream of the Rhb1-TOR pathway and is responsible for low-nitrogen-mediated filamentation by connecting with the Mep2-Ras1-PKA/MAPK pathway. Mep2 is a transmembrane permease responsible for ammonium uptake, and its cytoplasmic tail is required to activate invasive filamentation in response to nitrogen depletion, presumably through the cyclic AMP (cAMP)/PKA pathway and the Cph1-mediated MAPK pathway (52, 53). CBC directly controls MEP2 expression (Fig. 1B and 3C), and the constitutively active mutants Mep2 ΔC440 and Ras1 G13V can restore the filamentation defect of the hap5Δ mutant (Fig. 5D and E). This observa-
FIG 6 CBC negatively regulates cellular aggregation by repressing expression of adhesin genes under conditions unfavorable for filamentation. (A) Wild type, \( hap43 \), and CBC mutants were inoculated into 1 ml YPD medium and incubated at 30°C for 1 day. Flocculated cells were dispersed by pipetting and subsequently reaggregated by gentle shaking. Finally, the reaggregated flocs were sedimented by pipetting off the supernatants. Results from two independent experiments are shown. Flocs were observed for the \( hap5 \), \( hap31 \), \( hap32 \), and \( hap31 \) mutants but not the \( hap43 \) and wild-type strains. (B) Microscopic examination of aliquots of the cells from panel A. (C) Flocculated cells from overnight cultures in YPD (pH 6.8), acidic YPD (pH 4.2), or glucose-free yeast extract-peptone (YP) medium were sedimented by standing at room temperature. The formation of large flocs, rapid clearance of cell suspensions, and adherence of cells to test tube side walls were observed in the \( hap5 \), \( hap31 \), \( hap32 \), and \( hap31 \) mutants. (D) Fluorescent wild-type cells coflocculated with the nonfluorescent \( hap5 \) cells. DIC, differential interference contrast. (E) Quantitative RT-PCR analysis of the adhesin genes \( HWP1 \), \( ECE1 \), \( ALS1 \), and \( ALS3 \). Cells were grown in YPD at 30°C for 5 h. Expression levels are displayed as means ± SD for at least two independent experiments. Deletion of CBC, including \( HAP5 \), both \( HAP32 \) and \( HAP31 \), or \( HAP2 \), led to derepression of the adhesin genes.
tion indicates that CBC acts upstream of the Mep2-Ras1-PKA/MAPK pathway. Moreover, we previously reported that Mep2-mediated filamentation is regulated by Rhb1-TOR (44). Taken together, the data suggest that CBC is a central signaling connection between the Rhb1-TOR and Mep2-Ras1-PKA/MAPK signaling cascades.

CBC regulates virulence-associated traits.

In this study, CBC contributed to *C. albicans* virulence, as shown in peritoneal infections of zebrafish and systemic infections of mice (Fig. 7; see Fig. S4 in the supplemental material). However, the virulence defects observed in CBC mutants seem to be small. Because the virulence of *C. albicans* results from the combination of multiple factors, it is difficult to simply explain the results from our infection models by using just the results of *in vitro* assays. Nevertheless, CBC did play roles in regulation of different virulence-associated traits. For example, CBC mutants diminished cellular fitness in low-iron environments, utilization of host proteins, and low-nitrogen-induced filamentation, whereas the CBC mutants elevated the expression of adhesin genes.

CBC may act as a global complex for multiple transcription factors in *C. albicans.*

In the proposed model shown in Fig. 8B, we hypothetically included many potential factors in CBC-mediated transcription based on previous studies. TOR signaling can promote the expression of *NRG1* and *TUP1*, which encode transcriptional repressors, to further repress expression of adhesin genes and consequently inhibit flocculation (50). Moreover, *MEP2* expression under low-nitrogen conditions is controlled by Gln3 and Gat1 (54), both of which also regulate *SAP2* expression by regulating the transcription factor Stp1 (55). Interestingly, deletion of *GLN3*, *GAT1*, or *STP1* increases the rapamycin resistance of *C. albicans* (36, 56), suggesting that these regulators may also act as part of the TOR signaling pathway.

In summary, this study establishes an explicit connection between CBC and the regulation of virulence traits in *C. albicans.*

**FIG 7** CBC contributes to *C. albicans* virulence in a zebrafish model of peritoneal infection. A total of $1 \times 10^8$ *C. albicans* cells were injected per fish ($n = 20$ per *C. albicans* strain). The *hap43Δ, hap5Δ, hap32Δ hap31Δ*, and *hap2Δ* mutants showed reduced virulence compared to the wild-type and reintegrated strains. **, $P < 0.005$; *, $P < 0.05$.

**FIG 8** Model for CBC-mediated transcriptional regulation in *C. albicans*. (A) Schematic representation of putative signaling cascades mediated by CBC for low-nitrogen-induced filamentation. (B) Proposed model showing that genes involved in distinct virulence traits of *C. albicans* are regulated by CBC. Thick arrows indicate a “major” role in transcriptional regulation, whereas thin arrows indicate a “minor” role. Hypothetical components in CBC-mediated transcriptional regulation are drawn with dashed lines.
Some of the virulence traits that are related to nutrient acquisition and dependent on CBC are well conserved (48, 57–60). However, other traits display coherent C. albicans-specific or, possibly, fungus-specific characteristics. This work not only provides novel insights into regulation of virulence traits in C. albicans but also suggests a potential direction toward understanding the role of CBC in other important human fungal pathogens.

ACKNOWLEDGMENTS

This work was supported by grants NSC100-2627-B-007-002 and NSC101-2311-B-007-010-MY3 (to C.-Y.L.) from the National Science Council (Taiwan).

We are grateful to Alistair J. P. Brown, Joachim Morschhäuser, Rajendra Prasad, and Yu-Ting Chen for generously providing strains and plasmids used in this study. We are thankful to Shu-Jen Chou and Shu-Hsing Wu for technical assistance with microarray data analysis and to the DNA Microarray Core Laboratory (Institute of Plant and Microbial Biology, Academia Sinica, Taiwan) for providing the GeneSpring software.

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