Genetic Analysis of NDT80 Family Transcription Factors in Candida albicans Using New CRISPR-Cas9 Approaches

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ABSTRACT
Ndt80 family transcription factors are highly conserved in fungi, where they regulate diverse processes. The human fungal pathogen Candida albicans contains three genes (NDT80, REP1, and RON1) that encode proteins with similarity to Saccharomyces cerevisiae Ndt80, although the homology is restricted to the DNA binding domain. To better understand their role in virulence functions, we used clustered regularly interspaced short palindromic repeat/CRISPR-associated gene 9 (CRISPR/Cas9) to delete the three NDT80-family genes. An ndt80Δ mutant showed strong defects in forming hyphae in response to serum or N-acetylglucosamine (GlcNAc), which was linked to the ability of Ndt80 to regulate the expression of RAS1, an upstream regulator of hyphal signaling. Conversely, the ndt80Δ mutant formed hyphal cells on glycerol medium, indicating that Ndt80 is not required for hyphal growth under all conditions. In contrast to our previously published data, a ron1Δ single mutant could grow and form hyphae in response to GlcNAc. However, deleting RON1 partially restored the ability of an ndt80Δ mutant to form hyphae in response to GlcNAc, indicating a link to GlcNAc signaling. REP1 was required for growth on GlcNAc, as expected, but not for GlcNAc or serum to induce hyphae. The ndt80Δ mutant was defective in growing under stressful conditions, such as elevated temperature, but not the ron1Δ mutant or rep1Δ mutant. Quantitative assays did not reveal any significant differences in the fluconazole susceptibility of the NDT80-family mutants. Interestingly, double and triple mutant analysis did not identify significant genetic interactions for these NDT80 family genes, indicating that they mainly function independently, in spite of their conserved DNA binding domain.

IMPORTANTCE
Transcription factors play key roles in regulating virulence of the human fungal pathogen Candida albicans. In addition to regulating the expression of virulence factors, they also control the ability of C. albicans to switch to filamentous hyphal growth, which facilitates biofilm formation on medical devices and invasion into tissues. We therefore used new CRISPR/Cas9 methods to examine the effects of deleting three C. albicans genes (NDT80, REP1, and RON1) that encode transcription factors with similar DNA binding domains. Interestingly, double and triple mutant strains mostly showed the combined properties of the single mutants; there was only very limited evidence of synergistic interactions in regulating morphogenesis, stress resistance, and ability to metabolize different sugars. These results demonstrate that NDT80, REP1, and RON1 have distinct functions in regulating C. albicans virulence functions.

KEYWORDS
Candida albicans, NDT80, REP1, RON1, hyphae, morphogenesis

The human fungal pathogen Candida albicans commonly lives as a commensal on mucosal surfaces of most healthy humans, but it can initiate life-threatening systemic infections in those who are immunocompromised. Thus, C. albicans infections

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New and improved CRISPR/Cas9 methods were used to delete all three NDT80 family genes from the human fungal pathogen Candida albicans.
are the fourth most common type of nosocomial bloodstream infection (1). The attributable mortality rate is about 40% in spite of recent advances in antifungal therapy (2, 3), and the emergence of strains that are resistant to antifungal drugs is a further challenge to delivering effective therapy (4). Therefore, it is important to define the mechanisms of \textit{C. albicans} pathogenesis in order to develop new therapies. One major factor that promotes \textit{C. albicans} infections is its ability to grow in different morphologies ranging from budding cells to long chains of hyphal or pseudohyphal cells (5). The ability of \textit{C. albicans} to grow as long filamentous hyphae is significant for its pathogenic potential as it promotes invasive growth into host tissues and biofilm formation (5, 6). Hyphal growth can be induced \textit{in vitro} by a variety of environmental stimuli, including serum, alkaline pH, CO$_2$, and N-acetylglucosamine (GlcNAc) (5, 7–9). Mutational analysis has identified a network of transcription factors (TFs) that are important for inducing the hyphal morphology. These TFs are also needed to induce a special set of genes during hyphal growth that encode factors needed for virulence, such as adhesin proteins and superoxide dismutase (5, 10–12).

One TF needed for hyphal growth in \textit{C. albicans} is Ndt80 (10). The Ndt80 family is significant because it is highly conserved across a large group of fungal species (Fig. 1A). The number of Ndt80-like genes varies in different fungi, ranging from zero (\textit{Schizosaccharomyces pombe}) and one (\textit{Saccharomyces cerevisiae}) to six (\textit{Fusarium oxysporum} strain HDV247) (13). They regulate diverse processes, including sexual

\textbf{FIG 1} Strategy for multiple deletion of NDT80-family genes using transient CRISPR/Cas9 and the SAT1-FLP system. (A) In \textit{Candida albicans} (Ca), there are three genes that encode proteins with similarity to \textit{S. cerevisiae} (Sc) Ndt80. The amino acid sequence similarity is restricted to the DNA binding domain. The phylogenetic analysis of putative Ndt80 family proteins in Ascomycota indicates that Ndt80-like proteins can be assigned to two groups. One superbranch contains direct orthologs of \textit{S. cerevisiae} Ndt80, such as Ca Ndt80 and Ca Ron1. Ca Rep1 clusters in the other superbranch, which features orthologs from Pezizomycotina. Colored stars indicate \textit{S. cerevisiae} Ndt80 and \textit{C. albicans} Ndt80 family proteins as follows. Color code: \textit{Candida} CTG clade, blue; \textit{Saccharomyces cerevisiae}, green; \textit{Pezizomycotina}, red. (B) The CAS9 gene and the sgRNA were expressed transiently after transformation and were not integrated into the genome. The sgRNA targets Cas9 protein to produce a double-strand break (DSB) at a defined target sequence. The double-strand breaks can be repaired by homology-directed recombination with the SAT1-FLP cassette DNA fragment, which has homology on the ends to the target gene, to create a homozygous deletion of the gene of interest (GOI). The SAT1-FLP cassette confers nourseothricin (NAT) selection and marker recycling. Marker excision of the SAT1 gene is mediated by the maltose-inducible FLP recombinase, leaving a single FLP recombinase target (FRT) site in place of the each GOI.
development, filamentation, drug resistance, virulence, and the response to nutrient stress (13–16). One well-studied paralog is S. cerevisiae Ndt80, which is a key regulator of meiosis and sporulation (17, 18). In species that encode multiple Ndt80 homologs, genetic analyses indicate they can carry out distinct functions (13). In A. nidulans, one Ndt80-like protein (XprG) is a positive regulator that controls expression of an extracellular protease, mycotoxin production, and programmed cell death induced by carbon starvation (15), similarly to N. crassa VIB-1, which is required for expression of genes involved in heterokaryon incompatibility programmed cell death and, like XprG, is a positive regulator of extracellular protease production (14, 19). A. nidulans NdtA and N. crassa FSD-1, representing another type of Ndt80-related factor, have greater sequence similarity to S. cerevisiae Ndt80 and are required for sexual reproduction. Deletion of a third Ndt80-like gene in N. crassa (N. crassa U04729 [NCU04729]) had no effect on sexual reproduction or on any other phenotypes tested (14). Recently, functional analysis in Trichoderma reesei showed that one of Ndt80 homologs is a key activator of the GlcNAc gene cluster that is essential for GlcNAc catabolism (20).

There are three NDT80 paralogs in C. albicans (NDT80, RON1, and REP1). NDT80 has been shown to promote resistance to various stress conditions and to have a role in hyphal growth (21–23). We previously reported that a mutant lacking RON1 displayed defects in growing on GlcNAc media and in inducing hyphae in response to GlcNAc (24), but we showed here that it is not required by the use of a new clustered regularly interspaced short palindromic repeat/CRISPR-associated gene 9 (CRISPR/Cas9) method for making gene deletions. The third paralog, REP1, was identified as a negative regulator of the expression of the drug efflux pump MDR1 (25). REP1 was also shown to be needed for growth on some sugars, such as GlcNAc and galactose (26), and is thought to act in part by recruiting Ngs1 to specific promoters to regulate histone acetylation (26). To compare the mutant phenotypes in the same strain background, and to determine whether the Ndt80-like TFs have overlapping functions, we constructed single, double, and triple mutant combinations. To circumvent the difficulties of creating homozygous mutations in the diploid C. albicans, we took advantage of recent developments in the application of CRISPR-Cas9 (27–31), including transient CRISPR-Cas9 and SAT1-Flipper (27, 32). The use of the SAT1-Flipper drug resistance marker enabled us to analyze mutant phenotypes in fully prototrophic strains such that their phenotypes were not affected by auxotrophies. Interestingly, the results showed that the functions of Ndt80-like TFs are largely independent despite the presence of the conserved DNA binding domain (DBD) in C. albicans.

RESULTS

Creation of gene deletion strains using transient CRISPR-Cas9 and SAT1-FLP systems. To investigate the genetic interactions within the NDT80 TF family, we constructed a set of mutants that contained homozygous deletion mutations for each TF, the three possible double mutants, and a triple mutant. The mutations were constructed using transient CRISPR-Cas9 and SAT1-FLP systems (Fig. 1B). In construction of single mutants, wild-type strain S. cerevisiae 5314 (SC5314) was transformed with Cas9 DNA, which is transiently expressed (27); a gene encoding a single guide RNA (sgRNA) to guide the CRISPR-Cas9 to a specific site in the target gene; and a repair template consisting of the recyclable SAT1-FLP cassette providing resistance to nourseothricin (NAT+) (32) flanked by regions of homology to the sequences surrounding the targeted open reading frame. We then created the double deletion mutants in the NAT+ versions of the single deletion mutants in which the NAT+ gene was excised. A triple deletion mutant of the NDT80 TF family was generated in the ron1Δ rep1Δ NAT+ double mutant (Table 1). The frequency of creation of homozygous deletion mutants was similar to previous results for each round of gene deletions in the construction of the triple mutant (27).

Growth on different carbon sources. Cells lacking NDT80 (C2_00140W) grew well on various carbon sources (Fig. 2A and B), similarly to the previous results (24). In fact, we found that the ndt80Δ mutant displayed slightly better growth on nonfermentable
The deletion mutant lacking rep1Δ showed a strong defect with respect to growth on the amino sugars GlcNAc and glucosamine. Rep1 was reported to regulate GlcNAc catabolic genes by recruiting Ngs1 to the promoters of the genes (26). However, the rep1Δ mutant was also unable to grow on galactose, indicating that it has broader roles in regulating metabolism (Fig. 2A). The deletion mutant lacking RON1 (CR_04250W) did not exhibit a significant defect in growth on the different carbon sources. This contrasts with our previously published data (24) suggesting that mutant ron1Δ was defective in growth on GlcNAc and glucosamine. The reasons for these differences are unclear. However, we note that one difference is that the previous ron1Δ mutant was constructed in a different manner, including the use of auxotrophic markers for selection.

We next examined the set of double and triple homozygous mutants to determine if combining different mutations led to synergistic or compensatory growth phenotypes. However, none were detected (Fig. 2). All double and triple mutants lacking REP1 (mutants rep1Δ ndt80Δ, ron1Δ rep1Δ, and ron1Δ rep1Δ ndt80Δ) did not grow well on galactose, GlcNAc, and glucosamine, similarly to the rep1Δ mutant. The multiple deletion mutants lacking NDT80 (mutants rep1Δ ndt80Δ, ron1Δ ndt80Δ, and ron1Δ rep1Δ ndt80Δ) all showed slightly better growth on nonfermentable carbon sources such as was seen for the ndt80Δ single mutant.

**Hyphal morphogenesis.** Cell morphology was first examined after growth under conditions where the wild-type cells predominantly grew as budding cells (minimal glucose medium) (Fig. 3). In contrast, microscopic observation revealed that the ndt80Δ mutants formed connected chains of elongated cells. The multiple deletion mutants of NDT80 (mutants rep1Δ ndt80Δ, ron1Δ ndt80Δ, and ron1Δ rep1Δ ndt80Δ) also showed a similar defect in morphology. To determine whether the mutant cells could form hyphae, they were induced in minimal medium containing either serum or GlcNAc (Fig. 3). As expected, the wild-type strain switched to forming filamentous cells under these conditions. Consistent with previous studies (34), the ndt80Δ mutant was strongly defective in hyphal growth as it instead formed short, elongated cells with a swollen appearance in the presence of the serum or GlcNAc.

Deletion of REP1 did not affect hyphal induction in serum (Fig. 3). The rep1Δ mutant could not be induced in GlcNAc like the other strains, since it failed to grow on GlcNAc as the sole carbon source (Fig. 2A). On the basis of the inability of rep1Δ cells to grow

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**Table 1 C. albicans strains used in this study**

| Strain | Short genotype | Parent | Genotype |
|--------|----------------|--------|----------|
| SC5314 | Wild-type strain | SC5314 | ndt80Δ::SAT1-FLIP/ndt80Δ::SAT1-FLIP |
| KH1493 | ndt80Δ | SC5314 | rep1Δ::SAT1-FLIP/rep1Δ::SAT1-FLIP |
| KH1494 | rep1Δ | SC5314 | ron1Δ::SAT1-FLIP/ron1Δ::SAT1-FLIP |
| KH1495 | ron1Δ | SC5314 | rep1Δ::SAT1-FLIP/rep1Δ::SAT1-FLIP |
| KH1496 | rep1Δ ndt80Δ | rep1Δ Nat* | rep1Δ::FRT/rep1Δ::FRT ndt80Δ::SAT1-FLIP/ndt80Δ::SAT1-FLIP |
| KH1497 | ron1Δ ndt80Δ | ron1Δ Nat* | ron1Δ::FRT/ron1Δ::FRT ndt80Δ::SAT1-FLIP/ndt80Δ::SAT1-FLIP |
| KH1498 | ron1Δ rep1Δ | ron1Δ Nat* | ron1Δ::FRT/ron1Δ::FRT rep1Δ::SAT1-FLIP/rep1Δ::SAT1-FLIP |
| KH1499 | ron1Δ rep1Δ ndt80Δ | ron1Δ rep1Δ Nat* | ron1Δ::FRT/ron1Δ::FRT rep1Δ::FRT rep1Δ::FRT ndt80Δ::SAT1-FLIP/ndt80Δ::SAT1-FLIP |
| KH1500 | ndt80Δ + NDT80 | ndt80Δ Nat* | ndt80Δ::FRT/ndt80Δ::FRT rps1::NDT80-CIp10::SAT1::RPS1 |
| KH1501 | rep1Δ + REP1 | rep1Δ Nat* | rep1Δ::FRT/rep1Δ::FRT rps1::REP1-CIp10::SAT1::RPS1 |
| KH1502 | ron1Δ + RON1 | ron1Δ Nat* | ron1Δ::FRT/ron1Δ::FRT rps1::RON1-CIp10::SAT1::RPS1 |
| KH1503 | ndt80Δ Nat* | ndt80Δ | ndt80Δ::FRT/ndt80Δ::FRT |
| KH1504 | rep1Δ Nat* | rep1Δ | rep1Δ::FRT/rep1Δ::FRT |
| KH1505 | ron1Δ Nat* | ron1Δ | ron1Δ::FRT/ron1Δ::FRT |
| KH1506 | ron1Δ rep1Δ Nat* | ron1Δ rep1Δ | ron1Δ::FRT/ron1Δ::FRT rep1Δ::FRT/rep1Δ::FRT |

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Carbon sources (glucose, lactate, and acetate) than the wild-type control. Interestingly, the ndt80Δ mutant showed filamentous hyphal cells emanating from colonies grown on the synthetic glycerol plates whereas other strains, including the wild-type SC5313 strain, formed smooth-edged colonies (Fig. 2C). This phenotype was surprising since Ndt80 has been reported to be required for hyphal growth in *C. albicans* (10, 12, 33, 34). These results indicate that Ndt80 can also act as a repressor of filamentous growth under some conditions. Cells lacking REP1 (CR_04250W) showed a strong defect with respect to growth on the amino sugars GlcNAc and glucosamine. Rep1 was reported to regulate GlcNAc catabolic genes by recruiting Ngs1 to the promoters of the genes (26). However, the rep1Δ mutant was also unable to grow on galactose, indicating that it has broader roles in regulating metabolism (Fig. 2A). The deletion mutant lacking RON1 (CR_04250W) did not exhibit a significant defect in growth on the different carbon sources. This contrasts with our previously published data (24) suggesting that mutant ron1Δ was defective in growth on GlcNAc and glucosamine. The reasons for these differences are unclear. However, we note that one difference is that the previous ron1Δ mutant was constructed in a different manner, including the use of auxotrophic markers for selection.

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| KH1494 | rep1Δ | SC5314 | ron1Δ::SAT1-FLIP/ron1Δ::SAT1-FLIP |
| KH1495 | ron1Δ | SC5314 | rep1Δ::SAT1-FLIP/rep1Δ::SAT1-FLIP |
| KH1496 | rep1Δ ndt80Δ | rep1Δ Nat* | rep1Δ::FRT/rep1Δ::FRT ndt80Δ::SAT1-FLIP/ndt80Δ::SAT1-FLIP |
| KH1497 | ron1Δ ndt80Δ | ron1Δ Nat* | ron1Δ::FRT/ron1Δ::FRT ndt80Δ::SAT1-FLIP/ndt80Δ::SAT1-FLIP |
| KH1498 | ron1Δ rep1Δ | ron1Δ Nat* | ron1Δ::FRT/ron1Δ::FRT rep1Δ::SAT1-FLIP/rep1Δ::SAT1-FLIP |
| KH1499 | ron1Δ rep1Δ ndt80Δ | ron1Δ rep1Δ Nat* | ron1Δ::FRT/ron1Δ::FRT rep1Δ::FRT rep1Δ::FRT ndt80Δ::SAT1-FLIP/ndt80Δ::SAT1-FLIP |
| KH1500 | ndt80Δ + NDT80 | ndt80Δ Nat* | ndt80Δ::FRT/ndt80Δ::FRT rps1::NDT80-CIp10::SAT1::RPS1 |
| KH1501 | rep1Δ + REP1 | rep1Δ Nat* | rep1Δ::FRT/rep1Δ::FRT rps1::REP1-CIp10::SAT1::RPS1 |
| KH1502 | ron1Δ + RON1 | ron1Δ Nat* | ron1Δ::FRT/ron1Δ::FRT rps1::RON1-CIp10::SAT1::RPS1 |
| KH1503 | ndt80Δ Nat* | ndt80Δ | ndt80Δ::FRT/ndt80Δ::FRT |
| KH1504 | rep1Δ Nat* | rep1Δ | rep1Δ::FRT/rep1Δ::FRT |
| KH1505 | ron1Δ Nat* | ron1Δ | ron1Δ::FRT/ron1Δ::FRT |
| KH1506 | ron1Δ rep1Δ Nat* | ron1Δ rep1Δ | ron1Δ::FRT/ron1Δ::FRT rep1Δ::FRT/rep1Δ::FRT |
on GlcNAc, it was suggested they might also be defective in inducing the transcriptional responses needed to form hyphae (26). In order to test this, the rep1Δ cells were induced in minimal GlcNAc medium supplemented with glucose to provide a source of energy. Although glucose can repress the induction of the GlcNAc transporter (35), the
low concentration of glucose used in these experiments (5 mM) did not inhibit the
hyphal induction by high levels of GlcNAc (50 mM). Interestingly, the rep1Δ mutant was
stimulated efficiently by GlcNAc to form hyphae under those growth conditions (Fig. 3).
Thus, our result demonstrated that Rep1 is not important for GlcNAc induction of
hyphal morphogenesis.

Although we previously reported that Ron1 was defective in responding to GlcNAc
to form hyphae in liquid medium (24), the ron1Δ mutant made with CRISPR/Cas9
approaches did not show any obvious defects in responding to GlcNAc or serum
(Fig. 3). Interestingly, we also did not detect any synergistic defects in double and triple
mutant combinations lacking NDT80 family TFs.

The mutant cells were also tested for the ability to undergo invasive hyphal growth
into agar in the presence of different types of media (Fig. 4). Control studies showed the
expected results, i.e., that there were essentially no detectable hyphae emanating from
the zone of growth for the fungal cells spotted onto minimal glucose medium. In
contrast, the wild-type cells formed robust invasive hyphae 5 days after spotting of the
cells onto the surface of serum, Spider, and GlcNAc agar plates. The ndt80Δ cells were
strongly defective in invasive growth, as there were no hyphal outgrowths seen even
after 5 days of incubation on serum and Spider media (Fig. 4). The ndt80Δ mutant
showed only rare hyphal outgrowths on agar containing GlcNAc. The rep1Δ mutant was
not defective in invasive growth into agar containing serum or Spider media. Although
the rep1Δ mutant appeared to be strongly defective for invasion into GlcNAc medium,
this was likely due to the poor growth of the rep1Δ mutant on GlcNAc as a sole carbon
source (Fig. 2A). The ron1Δ mutant did not show an obvious defect in invasive hyphal
growth on agar containing GlcNAc, in contrast to our previous result (24). However, the
agar invasion studies revealed an interesting genetic interaction between NDT80 and
RON1. Deletion of RON1 partially rescued the hyphal defect of the ndt80Δ mutant on
GlcNAc (see the arrow in Fig. 4), indicating a role for RON1 in GlcNAc regulation.

Resistance to stress. We used a disk diffusion assay to measure the sensitivity of the
mutant cells to the antifungal drugs amphotericin B and fluconazole. To quantitatively
measure the drug sensitivity, we calculated the average radius in millimeters to the
point where 50% growth reduction occurred (RAD50) using diskImageR image analysis
software (see Materials and Methods). Statistical analysis showed that the single and
double deletion mutants of NDT80 family were similar to the wild type in amphotericin
B sensitivity (Fig. 5). The triple deletion mutant (mutant ron1Δ rep1Δ ndt80Δ) showed a statistically significant increase in sensitivity to amphotericin B, but the magnitude of difference was minor. Although it has been reported that Ndt80 and Rep1 are involved in fluconazole susceptibility (21, 22, 25); surprisingly, our disk diffusion assays did not reveal any significant differences in the fluconazole susceptibility of the mutants compared to the wild type. However, we observed that all the deletion mutants lacking NDT80 failed to show the trailing growth around the fluconazole disks that was seen for the wild-type strain (Fig. 5). Trailing growth (also called tolerance) is due to the slow growth of subpopulations of cells at antifungal drug concentrations above the MIC (36).

FIG 4 NDT80 is needed for invasive hyphal growth into agar. The cells indicated at the top were spotted onto the agar plates listed on the left. Serum was present in the medium at 15% (vol/vol), and GlcNAc was present at 2.5 mM. The plates were incubated at 37°C and then photographed after 5 days to record the extent of invasive growth emanating from the edges of the colonies. The deletion mutants lacking NDT80 (mutants ndt80Δ, rep1Δ ndt80Δ, ron1Δ ndt80Δ, and ron1Δ rep1Δ ndt80Δ) were strongly defective in invasive hyphal growth into agar media containing serum, Spider, or GlcNAc. Note that the ron1Δ ndt80Δ double mutant showed an improved ability to form hyphae on GlcNAc (arrow). The deletion mutants of REP1 (mutants rep1Δ, rep1Δ ndt80Δ, ron1Δ rep1Δ, and ron1Δ rep1Δ ndt80Δ) grew poorly and were not detectably induced on GlcNAc. Scale bars indicate 1 mm.

FIG 5 Sensitivity of mutants to antifungal drugs. The sensitivity to amphotericin B and fluconazole was determined by a disk diffusion assay in which cells were spread onto the surface of an RPMI 1640 medium plate and then filter discs containing 25 μg of the drugs were placed on the surface of the plate. The ron1Δ rep1Δ ndt80Δ triple mutant was slightly more sensitive to amphotericin B (AMB) than the wild-type SC5314 strain but was not more sensitive to fluconazole (FLC). None of the mutants showed significant differences in the zone of 50% growth inhibition (RAD50) in fluconazole compared to the wild-type strain. However, the deletion mutants lacking NDT80 (mutants ndt80Δ, rep1Δ ndt80Δ, ron1Δ ndt80Δ, and ron1Δ rep1Δ ndt80Δ) did not show the trailing growth around the fluconazole disks that was seen for the other strains. The plates were incubated at 30°C for 48 h and then photographed. Image analysis software was used to measure the zone of growth inhibition to determine the average radius that corresponded to a 50% growth reduction (RAD50). Double asterisks show statistically significant differences ($P < 0.01$) from the wild-type strain based on a Student’s $t$ test.
Because these “tolerant” cells continue to divide in the presence of antifungals, they might contribute to the persistence and/or recurrence of fungal infections (37). Rosenberg demonstrated that fluconazole tolerance was clearly distinct from fluconazole susceptibility measured as MIC in C. albicans (37). Therefore, our result indicates that deletion of NDT80 reduced drug tolerance of C. albicans without affecting the overall susceptibility to fluconazole.

The mutant cells were also tested for resistance to other stress conditions (Fig. 6). The ndt80Δ mutant grew poorly in Congo red, which binds and weakens the cell wall. The ndt80Δ mutant also showed sensitivity to the detergent SDS, which likely acts by disrupting the plasma membrane. These results indicate that deletion of NDT80 resulted in defects in cell wall and plasma membrane integrity. A previous study reported that an ndt80Δ mutant showed slow growth at 42°C (23). Although we could not see a growth defect clearly at 42°C, the ndt80Δ mutant showed a very strong growth defect at 44°C (Fig. 6). Deletion of REP1 or RON1 did not appear to further enhance these phenotypes of the ndt80Δ mutant; the double and triple deletion mutants lacking NDT80 (mutants rep1Δ ndt80Δ, ron1Δ ndt80Δ, and ron1Δ rep1Δ ndt80Δ) showed the same phenotypes as the ndt80Δ single mutant.

Ndt80 regulates transcription of RAS1. Given that Ndt80 has been found via chromatin immunoprecipitation (ChIP) to bind upstream of RAS1 during biofilm formation (10) and that Ras1 is known to play a key role in the cAMP signaling that leads to hyphal growth, we investigated whether Ndt80 regulates RAS1 expression. Using quantitative real-time PCR (qRT-PCR), we determined ratios of RAS1 expression to ACT1 expression in wild-type SC5314, ndt80Δ, and NDT80-complemented cells grown under hypha-inducing conditions. We observed that both ndt80Δ strains tested had significantly reduced RAS1/ACT1 ratios compared to SC5314 (Fig. 7). Complementing NDT80 back into an ndt80Δ strain restored RAS1 expression to nearly wild-type levels (Fig. 7). Taken together, these results suggest that Ndt80 was a positive regulator of RAS1 expression under the conditions tested.

**DISCUSSION**

Use of a recyclable marker system for CRISPR-Cas9 analysis of the NDT80 family. We further improved the previously reported transient CRISPR-Cas9 system (27) by using a SAT1-FLP system (32) for marker recycling. In this transient CRISPR system,
Cas9 and sgRNA are transiently expressed from linear DNA fragments that are not stable in the cells, thereby avoiding integration of CRISPR/Cas9 into the *C. albicans* genome and the potential problems that could arise from long-term expression of this enzyme (27). The incorporation of a fusion PCR method for generating new sgRNAs streamlined the process of making gene deletions. However, the original system was not adequate to produce multiple deletion mutants since it did not use recyclable markers (27). Huang and Mitchell solved this problem by surrounding the selectable markers with direct repeats, enabling CRISPR-directed marker recycling. In their approach, two auxotrophic marker genes could be used to sequentially delete three or more genes in the same strain (30). Another approach for recycling selectable markers was used by Nguyen et al. in which CRISPR-Cas9 and a selectable marker were integrated in tandem into the genome and were then excised after confirming that the expected genome editing event had occurred (31). Although this method enables rapid gene editing, the initial stable genome integration results in constitutive expression of CRISPR-Cas9 system that could induce off-target mutations in the genome. In the present study, we therefore combined the use of transient CRISPR/Cas9 and a recyclable SAT1-FLP marker for selection. This approach allowed us to construct single, double, and triple mutant combinations of the three *NDT80* family genes in a wild-type strain background with no concerns about interference due to auxotrophic markers used for selection and the fact that transient CRISPR/Cas9 system does not generate a permanent disruption of a genomic locus.

**Roles of Ndt80 family transcription factors in filamentous hyphal growth.** Using the new CRISPR/Cas9 approaches, we analyzed the effects of deleting the three *NDT80*-family genes in the same strain background without the use of auxotrophic selection markers. The *ndt80Δ* mutant was defective in forming hyphae in response to the strong inducers serum and GlcNAc, as expected. Interestingly, we found that Ndt80 regulates the expression of *RAS1*, which is a key upstream component of the cAMP signaling pathway that is needed for hyphal growth (Fig. 7). These results are consistent with ChIP sequencing (ChIP-Seq) data, which have shown that Ndt80 binds to the

![FIG 7](image-url) Ndt80 regulates expression of RAS1. Deletion of *NDT80* resulted in significantly reduced *RAS1* expression compared to wild-type strain SC5314. Complementation of an *ndt80Δ* mutant with *NDT80* restored *RAS1* expression to nearly wild-type levels. Cells were cultured under hypha-inducing conditions (YNBNP medium, pH 7.0, 37°C) for 1 h prior to RNA extraction and cDNA synthesis for qRT-PCR analysis. Values are shown as ratios of *RAS1* transcription to *ACT1* transcription. Asterisks show statistically significant differences (*, *P* < 0.05; **, *P* < 0.01) from the wild-type strain based on one-way analysis of variance (ANOVA) with multiple comparisons.
promoter region of the RAS1 gene (12). However, the ndt80Δ mutant appears to have a stronger hyphal defect than a ras1Δ mutant (38, 39) indicating that Ndt80 must also regulate other aspects of hyphal growth. In addition, the ndt80Δ mutant showed abnormal cell morphology under budding conditions, which is not seen for ras1Δ mutants (38, 39). Interestingly, Ndt80 was not essential for filamentous growth under all conditions as ndt80Δ cells grown on glycerol medium instead of glucose formed hyphae (Fig. 2B and C). This contrasting phenotype implies that the requirement for Ndt80 in filamentous growth changes depending on the environmental condition.

In this study, deletion of RON1 did not reveal significant phenotypes, in contrast to our previous study of a ron1Δ mutant made with a different approach that indicated a role for RON1 in regulating GlcNAc signaling (24). However, the ndt80Δ ron1Δ double mutant had an improved ability to form hyphae on GlcNAc medium, which is consistent with a role for RON1 in GlcNAc regulation. This phenotype suggests that RON1 can act as a negative regulator of filamentous growth response to GlcNAc in the absence of NDT80. Chip-Seq performed by Nocedal et al. showed that Ron1 binds to many fewer genomic regions but that all those sites are also bound by Ndt80 (12). This suggests that Ron1 may influence expression of a subset of genes regulated by Ndt80.

Our results indicated that Rep1 was not needed for hyphal induction when cells were stimulated by GlcNAc (Fig. 3). This contrasts with a suggestion from the previous study by Su et al. (26). They predicted that Rep1 could be involved in stimulating hyphal growth because it is needed to induce GlcNAc catabolic genes by recruiting the histone acetyltransferase, Ngs1, to the promoter region of these GlcNAc genes (26). However, when the rep1Δ mutant was supplied with an alternative sugar to promote growth, it could be induced to form hyphae in response to GlcNAc (Fig. 3). This is consistent with the previous finding that GlcNAc induction of hyphal morphogenesis in C. albicans is not dependent on its metabolism (40).

**Roles of NDT80 family transcription factors in response to drugs and stress.** Previous studies reported that the ndt80Δ mutant was sensitive to fluconazole whereas the rep1Δ mutant was resistant to fluconazole (22, 25). The authors of those studies used spot assays, which do not quantify the changes in drug sensitivity. To compare fluconazole sensitivities in a quantitative manner, we used disk diffusion assays, which showed that three different deletion mutants lacking the NDT80 family genes were not significantly different from the wild-type strain (Fig. 5). Instead, we found that the ndt80Δ mutant was distinct in that it did not show the typical trailing growth that is seen with the wild-type strain. Rosenberg et al. demonstrated that this type of drug tolerance is clearly distinct from susceptibility/resistance measured as MIC or halo size (37). We also found that the ndt80Δ mutant was susceptible to Congo red, SDS, and high temperature (Fig. 6). These phenotypes suggest that Ndt80 has multiple functions in stress response under various conditions.

**Limited genetic interactions among the members of the NDT80 family.** We hypothesized that the three NDT80 family genes might display strong genetic interactions due to the similarity of their DBDs. Surprisingly, in spite of the fact that a previous study indicated that Ron1 binds to a subset of Ndt80 binding sites in the genome, we observed only a weak genetic interaction between ndt80Δ and ron1Δ for GlcNAc-induced hyphal growth and did not detect other evidence for genetic interactions. Furthermore, deletion of REP1 did not alter the hyphal defect of the ndt80Δ mutant or show a genetic interaction with ron1Δ. Similarly, deletion of RON1 or NDT80 did not change the growth defect of the rep1Δ mutant on galactose, glucosamine, or GlcNAc, which is consistent with the fact that Ndt80 and Ron1 did not bind to the promoters of GlcNAc catabolic genes (12) that are reported to be bound by Rep1 (26). These results suggest that the three NDT80 family TFs are functionally independent and that their regulatory networks do not affect each other. A further sign of this is that Nocedal et al. did not detect the binding of Ndt80 or Ron1 to each other's promoter regions or to the promoter region of REP1 (12). Consistent with this, even the ron1Δ rep1Δ ndt80Δ triple mutant displayed a phenotype similar to the combined phenotypes of the corresponding single mutants.
The failure to detect genetic interactions for the NDT80 family is in sharp contrast to the results of analysis of other genetic networks, such as the transcription factors involved in biofilm formation (Bcr1, Brg1, Efg1, Ndt80, Rob1, and Tec1) (33). In the latter case, many double mutants showed synergistic defects and were severely defective in biofilm formation. The biofilm network showed properties of a highly interdependent network that is susceptible to genetic perturbation (10, 33). It is interesting that Ndt80 is highly interconnected with other transcription factors in the biofilm network whereas it is largely independent of the other NDT80 family transcription factors in spite of their conserved DBD. The roles for the three NDT80-family genes in C. albicans therefore appear to be similar to the function of the multiple NDT80-family genes found in filamentous fungi. The NDT80 family TFs in filamentous fungi fall into two main groups; one group has functions in sexual development, and the other has various functions in metabolism, including GlcNAc catabolism (13–15, 19). Taken together, these results help us to understand the role of the highly conserved NDT80 family of transcription factors in fungi.

MATERIALS AND METHODS

Strains and culture conditions. The genotypes of the C. albicans strains that were used are described in Table 1. All strains were stored in 15% glycerol stocks at −80°C. All C. albicans strains were streaked from −80°C onto YPD (1% yeast extract, 2% peptone, 2% glucose) plates every week and incubated at 30°C. C. albicans transformants were selected on YPD plus 200 μg/ml nourseothricin (NAT; Werner BioAgents) for nourseothricin-resistant (Nat') isolates. To obtain nourseothricin-sensitive (Nat') derivatives, transformants were grown overnight in maltose medium (1% yeast extract, 2% peptone, 2% maltose). Cells were then spread on YPD plates containing 25 μg/ml of NAT and incubated for 2 days at 30°C. Nat' colonies were screened by their colony size, which was smaller than that of their Nat' parental strains.

Fungal strains were tested for growth on different sugars by spotting dilutions of cells on synthetic agar medium containing 0.67% yeast nitrogen base with ammonium sulfate and the indicated carbon source. Fungal strains were also tested for resistance to different stress conditions by spotting dilutions of cells on YPD agar medium containing 140 μM Congo red or 0.06% SDS or by incubation at 44°C. Strains were grown overnight in YPD with shaking and adjusted to 2 × 107 cells/ml, and then 5-fold dilutions of cells were prepared. A 3-μl aliquot of each dilution was then spotted onto the indicated type of plate, and the plates were incubated at 30°C or 44°C as indicated.

The ability to form hyphae in liquid media was analyzed with cells grown overnight at 30°C in YPD. The cells were then diluted to 2 × 106 cells/ml into synthetic medium containing 50 mM glucose. After 3 h of incubation at 30°C, hyphal growth was induced by a shift in temperature to 37°C in combination with 15% bovine serum. For N-acetylglucosamine (GlcNAc) induction, the cells were diluted into synthetic medium containing 50 mM GlcNAc and incubated at 37°C. Glucose (5 mM) was added to the GlcNAc medium to support growth of the rep1Δ strains.

Invasive hyphal morphogenesis was analyzed by spotting 3 μl of serial dilutions of cells on an agar plate with the indicated type of medium and then incubating at 37°C. Cells were induced on synthetic medium containing 2.5 mM GlcNAc, agar medium containing 15% bovine serum, or Spider medium. The morphology of the cells at the edge of the zone of growth was photographed to record the extent of invasive hyphal growth into the agar.

Assaying susceptibility to antifungal drugs with disk diffusion assays. C. albicans cells were grown overnight in YPD, and then 2.5 × 106 cells were spread onto 20-ml RPMI 1640 agar plates. Stock solutions of amphotericin B (Sigma-Aldrich Corp.) and fluconazole (Sigma-Aldrich Corp.) were prepared at 10 mg/ml in dimethyl sulfoxide (DMSO). A 2.5-μl volume of the stock solution (25 μg) was spotted onto 6-mm-diameter paper disks and allowed to dry in the dark. A single paper disk was placed in the center of each plate, and then the plates were incubated at 30°C for 48 h and each plate was photographed individually. Analysis of the disk diffusion assay was done using the diskImageR package from the R library. The diskImageR analysis measured pixel intensity corresponding to cell density and calculated the average radius in millimeters to the point where 50% growth reduction occurred (RAD50) (41).

Macroscopic and microscopic analysis of cells. Cells were allowed to grow on solid agar media for the indicated number of days and then photographed. Unless otherwise noted, all spot assays were completed as at least three independent replicates, and a representative data set is shown. To record the extent of invasive hyphal growth into the agar, the cells at the edge of the zone of growth were photographed using an Olympus BH2 microscope equipped with a 4× objective and a Zeiss AxioCam digital camera. To image the morphology of cells under hypha-inducing conditions, the cells in liquid medium were concentrated by centrifugation and then photographed using differential interference contrast (DIC) optics. Photographic images were captured using a Zeiss Axio Observer 7 inverted microscope equipped with a 100× objective and a Zeiss AxioCam 702 digital camera.

DNA and plasmids. The single, double, and triple mutant strains of NDT80 family were created using transient expression of CRISPR-Cas9 to facilitate the homozygous deletion of a target gene. The methods were performed essentially as described previously (27). We used 20-bp target sequences of the sgRNA,
as reported previously by Vyas et al. (28), to delete ND780 (target sequence GCATGCCCGATTGATAGA), REP1 (TGTAGTGGACCATCTCCG), and RON1 (TCTGCCACATTAAGCAC). Gene deletion PCR constructs were synthesized using plasmid pGR-NAT as the template. The pGR-NAT plasmid has a full cassette of SAT1 flipper (32). The primers were designed to include 60 to 80 bases of homology to the sequences upstream or downstream from the target gene. PCR was conducted with Ex Taq in accordance with the manufacturer's instructions (TaKaRa Bio, Inc.).

The complemented strains of the ND780 family were constructed by integrating a copy of the wild-type gene sequence into the genome. The ND780, REP1, and RON1 plasmids were constructed by PCR amplification of genomic DNA from 1 kb upstream of the start codon and 0.5 kb downstream of the stop codon of the ND780, REP1, and RON1 genes, respectively. PCR was conducted with Phusion in accordance with the instructions of the manufacturer (New England Biolabs, Inc.). The primers were designed to include 20 bases with homology to Clp10-SAT1, a SAT1-marked version of plasmid Clp10 (42, 43). The DNA fragments were then inserted at EcoRI restriction site of the Clp10-SAT1 plasmid using Gibson assembly master mix (New England Biolabs, Inc.). The constructed plasmids were linearized by the use of StuI and integrated into the RPS1 locus of the fungal strains.

**Fungal transformation.** PCR products and linearized plasmids for transformation were purified and concentrated by the use of QiAquick PCR purification kit (Qiagen N.V.). Electroporation was used to introduce the DNA into the cells in order to obtain optimal transformation efficiency. The electrocompetent fungal cells were prepared following a previously described method (44). A cell suspension (40 μl) was added to aliquoted DNA, placed in electroporation cuvettes, and electroporated on a Bio-Rad Gene Pulser at 1.5 kV. One milliliter of 0.5×YPD containing 1 M sorbitol was added immediately to the cuvette, and the mixture was incubated for 3 h at 30°C before plating onto selective media was performed. Nat+ transformants were selected, and PCR genotyping of the transformants verified that both copies of the target gene had been deleted. Marker excision was mediated by the maltose-inducible FLP recombinase, and Nat+ cells were screened as described above (32).

**qRT-PCR.** Fungal cells were grown overnight in YPD, washed once with phosphate buffer (pH 7.0), and resuspended in phosphate buffer. Approximately 4.5 × 10^6 cells were inoculated in 5 ml YNB medium (0.67% YNB, 0.2% glucose, 25 mM phosphate buffer, 5 mM MgCl2) at pH 7.0 and incubated at 37°C for 1 h. RNA was extracted using a MasterPure yeast RNA purification kit (Epicentre). DNA contamination was removed by the use of Turbo DNA-free kit (Invitrogen). cDNA was synthesized from RNA using a random hexamer primer (IDT) and GC rich template. qRT-PCR was performed on a CFX96 real-time system (Bio-Rad), using SsoFast Evergreen Supermix with the indicated primers to detect transcripts. Thermocycler conditions were as follows: 95°C for 30 s followed by 40 cycles of 95°C for 5 s, 65°C for 3 s, and 95°C for 5 s. RAS1 transcripts were normalized to ACT1 transcripts.

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