EC Accepts, published online ahead of print on 22 January 2010
Eukaryotic Cell doi:10.1128/EC.00325-09
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Role of the transcription factor CaNdt80p in cell separation, hyphal growth and virulence in *Candida albicans*

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Running title: CaNdt80p regulates morphogenesis in *Candida albicans*

Key words: *Candida albicans*, Transcription factor, Cell separation, Morphogenesis, virulence.

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Summary

The NDT80/PhoG transcription factor family includes ScNdt80p, a key modulator of the progression of meiotic division in *Saccharomyces cerevisiae.* In *Candida albicans* a member of this family, CaNdt80p, modulates azole sensitivity by controlling expression of ergosterol biosynthesis genes. We previously demonstrated that CaNdt80p promoter targets, in addition to *ERG* genes, were significantly enriched in genes related to hyphal growth. Here we report that CaNdt80p is indeed required for hyphal growth in response to different filament-inducing cues and for the proper expression of genes characterizing the filamentous transcriptional program. These include noteworthy genes encoding cell wall components such as *HWP1*, *ECE1, RBT4* and *ALS3*. We also show that CaNdt80p is essential for the completion of cell separation through the direct transcriptional regulation of genes encoding the chitinase Cht3p and the cell wall glucosidase Sun41p. Consistent with their hyphal defect, *ndt80* mutants are avirulent in a mouse model of systemic candidiasis. Interestingly, based on functional domain organization, CaNdt80p seems to be a unique regulator characterizing fungi from the CTG clade within the Saccharomycotina subphylum. Therefore this study revealed a new role of the novel member of the fungal NDT80 transcription factor family as a regulator of cell separation, hyphal growth and virulence.

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Introduction

Candida albicans is an opportunistic pathogen responsible for various non life-threatening infections such as oral thrush and vaginitis, and accounts for more than half of all *Candida* infections (21, 40). This pathogen is also a major cause of morbidity and mortality in bloodstream infections, especially in immunosuppressed individuals. In addition, *C. albicans* can colonize various biomaterials and readily forms dense biofilms that are resistant to most antifungal agents. The ability of this fungus to switch from yeast to filamentous forms (true hyphae or pseuohyphae) is a crucial determinant for host invasion and thus virulence (1). Hyphal growth can be initiated by different environmental cues such as temperature, pH or nutrient availability (1). Consequently, morphological switching implies a complex interplay of various sensing and signal transduction pathways as well as transcriptional regulatory networks stimulating or repressing hyphal formation. Deciphering the molecular mechanisms that underlie this morphological switch is currently of high interest. Despite the large number of studies in recent year, the molecular determinism of *C. albicans* morphogenesis is still not fully understood.

The NDT80/PhoG transcription factor (TF) family includes the DNA-binding meiosis-specific protein ScNdt80p, a key modulator of the progression of the meiotic divisions in the yeast *Saccharomyces cerevisiae* (15, 33). This family also includes VIB-1, which is a regulator of conidiation in *Neurospora crassa* (41) and shares a region of similarity to PhoG, a possible phosphate non-repressible acid phosphatase in *Aspergillus nidulans* (23). Structural studies revealed that this family is related to the Ig-fold family of

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transcription factors, which includes the human TFs p53, NF-κB, STAT, AML-Runt, and the Rel subfamilies (20, 24). All of these Ig-fold proteins bind DNA in a similar manner using loops and other features at one end of the β-sandwich. In *C. albicans* a member of this family, named CaNdt80p (orf19.2119), has recently been identified as a key modulator of azole sensitivity dues to its participation in the control of ergosterol biosynthesis gene expression (36). Genome-wide occupancy using chromatin immunoprecipitation coupled with high-density tiling arrays showed that, in addition to *ERG* genes, this TF bound a large number of gene promoters with diverse biological functions such as cell wall, hyphal growth, carbohydrate metabolism and mitotic cell cycle. Additionally, *de novo* motif analysis of CaNdt80p-bound promoters revealed that, as in *S. cerevisiae*, this regulator bound to the middle sporulation element 5'-gNCRCAAAY-3' in *C. albicans* (where lowercase letters indicate semiconserved residues, R indicates a purine, N indicates any nucleotide, and Y indicates either a thymine or a cytosine).

The finding that CaNdt80p occupies the promoter regions of 23% of *C. albicans* genes suggests that this TF might control other biological processes in addition to drug sensitivity (6, 36). Since CaNdt80p targets were significantly enriched in genes related to hyphal growth, this prompted us to study its potential role in morphological switching and host invasion. In this study, we have continued to elucidate the multiple functions of CaNdt80p in *C. albicans* by demonstrating its central role in regulating cell separation, hyphae differentiation and virulence. Interestingly, based on its functional domain

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organization, CaNdt80p seems to be a unique TF characterizing fungi from the CTG clade within the Saccharomycotina subphylum.

Materiels and methods

C. albicans strains, plasmids and media

Strains used in this study are listed in Table 1. For general propagation and maintenance conditions, the strains were cultured at 30°C in yeast-peptone-dextrose (YPD) medium supplemented with uridine (2% Bacto peptone, 1% yeast extract, 2% dextrose, and 50 µg/ml uridine, with the addition of 2% agar for solid medium). Cell growth, transformation and DNA preparation were carried out using standard yeast procedures. For filamentation assays, cells were grown at 37°C in YPD supplemented with either 10% of fetal bovine serum (Invitrogen) or 2.5 mM of N-acetyl-D-glucosamine (Sigma) or in M199 medium (Sigma) buffered with 150 mM HEPES to pH 8.0. For growth under hypoxic conditions, cells were spotted on YPS (2% Bacto peptone, 1% yeast extract, 2% sucrose, 2% agar) plates and incubated in an anaerobic chamber (Oxiod HP0011A) at 37°C. The chamber was flushed daily with nitrogen to remove oxygen and any by-products.

Cell separation defects were assessed as described previously by (11) except that more than 500 cells were counted for each strain.

To overexpress *CHT3* and *SUN41* in the null mutant *ndt80*, ORFs of each gene were amplified from genomic DNA using the two sets of primers Cht3F1/Cht3R1 and Sun41F1/Sun41R1 (Table 2), respectively. The PCR fragments were digested with

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restriction enzymes MluI and NheI and cloned in the same sites of the CIp-ACT1-CYC vector (2). The plasmid was sequenced to confirm the integrity of the genes. Plasmids CIp-ACT1-CHT3 and CIp-ACT1-SUN41 were digested with the StuI restriction enzyme and used to transform the *ndt80* mutant strain (AS32). The absence of aneuploidy was confirmed in the overexpressing mutants using comparative genome hybridization as described by (36).

Gene Expression Profiling

For gene expression profiling under yeast form, saturated overnight cultures of the wild-type (BWP17) and *ndt80/ndt80* strain *AS31* were diluted to a starting OD_{600} of 0.1 in 50 ml fresh YPD and grown at 30°C to an OD_{600} of 0.8. Hyphae were induced by growing *Candida* cells in YPD+ 10% fetal bovine serum (FBS) at 37°C to an OD_{600} of 0.8. Cultures were harvested by centrifugation at 3000 x g for 5 min, and the pellet rapidly frozen in liquid nitrogen.

To extract RNA from cells, samples stored at -80°C were placed on ice and RNeasy buffer RLT was added to pellets at a ratio of 10:1 [vol/vol] buffer/pellet. The pellet was allowed to thaw in the buffer while vortexing briefly at high speed. The resuspended pellet was placed back on ice and divided into 1 ml aliquots in 2 ml screw cap microcentrifuge tubes containing 0.6 ml of 3 mm diameter acid-washed glass beads. Samples were homogenized 5 times, 1 min each, at 4200 RPM using Beadbeater. Samples were placed on ice for 1min after each homogenization step. After the homogenization the Qiagen RNeasy protocol was followed as recommended. Total RNA

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samples were eluted in RNAse free H_2O. RNA quality and integrity were assessed using an Agilent 2100 bioanalyzer.

cDNA labelings and microarray hybridizations were performed as previously described (30). Briefly, 20 µg of total RNA was reverse transcribed using oligo(dT)21 in the presence of Cy3 or Cy5-dCTP (Invitrogen) and Superscript III reverse transcriptase (Invitrogen). Thereafter, template RNA was degraded by adding 2.5 units RNase H (USB) and 1 µg RNase A (Pharmacia) followed by incubation for 15 min at 37°C. The labeled cDNAs were purified with QIAquick PCR Purification Kit (Qiagen). Prior to hybridization Cy3/Cy5-labeled cDNA was quantified using a NanoDrop ND-1000 UV-VIS spectrophotometer (NanoDrop) to confirm dye incorporation. Pre-hybridization and hybridization solutions consisted of DIG Easy Hyb solution (Roche Diagnostics, Mannheim, Germany) with 0.45% salmon sperm DNA and 0.45% yeast tRNA. The hybridization was carried out at 42°C for 20 h in a SlideBooster Hyb chamber SB 800 (Advalytix, Brunnthal, Germany) with a regular microagitation of the sample. Slides were washed once in 1.0% SSC, 0.2% SDS at 42°C for 5 min, twice in 0.1% SSC, 0.2% SDS at 42°C for 5 min, once in 0.1% SCC at 24°C for 5 min, followed by four rinses in 0.1% SSC. Chips were air dried before being scanned using a ScanArray Lite microarray scanner (Perkin Elmer). Microarray data was analyzed in GeneSpring GX v7.3 (Agilent Technologies) and genes with statistically significant changes in transcript abundance were identified using a Welch *t-test* with a False Discovery Rate (FDR) of less than 5%.

Expression analysis by real-time quantitative PCR

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For quantitative real-time PCR (qPCR), cDNA was synthesized from 5 µg of total RNA using the Invitrogen reverse-transcription system (50 mM Tris-HCl, 75 mM KCl, 5 mM DTT, 3 mM $MgCl_2$, 400 nM oligo$(dT)_{15}$, 20 ng random octamers, 0.5 mM dNTPs, 200 units Superscript III reverse transcriptase). The mixture was incubated for 60 min at 50°C. cDNAs were then treated with 2U of RNase H (Promega) for 20 min at 37°C followed by heat inactivation of the enzyme at 80°C for 10 min. Aliquots were used for qPCR, which was performed using the Mx3000P QPCR System (Agilent) with the QuantiTect SYBR Green PCR master mix (Qiagen). Cycling was 10 min at 95°C followed by 40 cycles (95°C, 10 s; 58°C, 15 s; 72°C, 15 s). Samples were done in triplicate and means were used for calculations. Fold changes were estimated by using the coding sequence of the *C. albicans ACT1* ORF as a reference. Fold enrichments of the tested coding sequences were estimated using the comparative ΔΔCt method as described by (14).

Virulence studies

Mouse studies were carried out as previously described (27). Briefly, 8-12 week-old B6 mice (Jackson Laboratories, Bar Harbor, ME) were inoculated via the tail vein with 200 µl of a suspension containing 3×10^5 *C. albicans* in PBS. Six mice, three females and three males, were used for each experimental group. Mice were closely monitored and those showing ruffled fur, hunchback and extreme lethargy were considered moribund and were euthanized. To determine fungal loads, kidneys from each mouse were removed aseptically and homogenized in 5 ml of PBS before plating on YPD plates containing chloramphenicol (34 µg/ml). The number of yeast colonies per kidney was determined

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and log-transformed. All experimental procedures involving animals were approved by the Biotechnology Research Institute Animal Care Committee, which operated under the guidelines of the Canadian Council of Animal Care.

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Results

Saccharomycotina CTG clade harbor two distinct paralogs of the NDT80/PhoG like TF family

Screening the *C. albicans* genome allowed us to identify two putative proteins with a conserved NDT80/PhoG like DNA-binding domain, corresponding to *ORF19.2119* (CaNdt80p) and *ORF19.513*. Analysis of their DNA binding domains (DBD) at the amino acid level revealed that they both share 55% similarity with the meiosis-specific transcription factor ScNdt80p of *S. cerevisiae*. Domain architecture analysis of the two *C. albicans* TF showed that *ORF19.513* had similar domain organization to ScNdt80p consisting of the DBD located in the N-terminal region followed by a putative activation domain at the C-terminal region (Fig.1A). In contrast, *ORF19.2119* exhibited a unique organization that is the opposite to that of orf19.513 and ScNdt80p (Fig. 1A).

Examination of ascomycetes genomes using BlastP program was performed in order to identify putative orthologs of NDT80/PhoG TF. Results show that all ascomycetes fungi have a single putative Ndt80p displaying a domain organization similar to that of ScNdt80p and orf19.513. The sole exception was the monophyletic CTG clade within the Saccharomycotina containing organisms that translate CTG as serine instead of leucine, which harbor an additional gene homolog with a domain organization similar to orf19.2119 (Fig. 1B).

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Deletion of *CaNDT80* affects cell separation

By combining genome-wide location and transcriptional profiling, we have previously revealed a key role of CaNdt80p in modulating azole sensitivity through the regulation of the expression of ergosterol (*ERG*) biosynthesis genes (36). In addition to *ERG* gene promoters, Ndt80p was found to bind a large number of gene promoters demonstrating that this regulator might operate in other biological processes. In order to further analyze other potential cellular functions controlled by CaNdt80p, we have monitored the yeast-growth morphology of cells missing both *ndt80* alleles when cultured in YPD at 30°C. As shown in Fig. 2A, microscopic observation revealed that *ndt80* cells showed an altered cell morphology corresponding to a defect in cell separation as well as an abnormal cell size compared to both WT and revertant strains. *ndt80* mutants consist of relatively swollen yeast cells forming chains connected by septa, as visualized by calcofluor white staining, along with a significant increase in the percentage of chains of cell with 3-4, 5-6 or more than 6 cells (Fig.2B).

CaNdt80p is required for the transcriptional activation of cell separation genes

To gain insight into the underlying molecular mechanism leading to the cell separation defect, we examined transcriptional differences between WT and *ndt80* cells growing in YPD at 30°C using whole-genome microarrays. Using a statistical significance analysis with an estimated false discovery rate of 5%, in addition to a cutoff of 1.5-fold, we identified 111 genes that require CaNdt80p for their proper expression, including 68 upregulated genes and 43 downregulated genes (Table S1).

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Our previous genome-wide location study demonstrated that, under a yeast promoting growth condition, CaNdt80p binds 1446 gene promoters (36). By cross-referencing this data with the list of Ndt80p-transcriptionally dependent genes, we were able to identify candidate CaNdt80p direct target promoters whose genes are transcriptionally regulated during the yeast growth phase (Fig.3A). Gene expression analysis indicated that some CaNdt80p-direct-targets were activated (46 genes), whereas others were repressed (25 genes) in the *ndt80* mutant. This finding suggests that this TF functions both as an activator and a repressor of gene expression.

Interestingly, among genes that the *ndt80* mutants failed to activate, we found the putative cell wall glycosidase *SUN41,* which is required for cell separation in *C. albicans* (11, 16). Additionally, we were also interested in the Cht3p chitinase, which showed an average reduction in transcript abundance of 4.5-fold in *ndt80/ndt80* mutants, although these levels of repression were not consistent enough for it to pass the threshold of statistical significance in our 4 replicates. Both the *SUN41* and *CHT3* gene promoters are bound by CaNdt80p (Fig. 3B), and their inactivation leads to a cell separation defect similar to that observed in *ndt80* mutants. This suggests that CaNdt80p could directly control the expression of genes implicated in cell separation completion and that the cell separation defect in *ndt80* mutant is the consequence of *CHT3* and/or *SUN41* depletion.

The *ndt80* cell separation phenotype is suppressed by overexpression of *CHT3* or *SUN41*

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In order to confirm that the cell separation defect observed in *ndt80* is related to the depletion of at least one of these two cell wall degradation genes, we sought to investigate if overexpression of either *CHT3* or *SUN41* could restore the WT phenotype in *ndt80* mutants. For this purpose the *CHT3* and *SUN41* ORFs were placed under the control of the *CaACT1* promoter and expressed in the *ndt80* background. Overexpression of these two genes was confirmed using real time quantitative PCR and the results demonstrated that the expression of *CHT3* and *SUN41* were significantly augmented compared to the WT strain (Fig. 4A). We then quantified the cell separation defect by counting the number of cells per chains in the overexpression mutants. As shown in Fig. 4B, the *ndt80* cell separation phenotype is reverted by overexpressing either *CHT3* or *SUN41*. Indeed, cells are found predominantly as single cells or as mother-daughter cells in overexpression mutants similarly to what was observed in the WT and revertant strains. This support that the cell separation defect in *ndt80* cells can be attributed to the depletion of *CHT3* and *SUN41*.

CaNdt80p is essential for hyphal growth in response to different filament-inducing conditions

The *ndt80* mutants were also tested for their ability to form hyphae under different environmental conditions. WT cells grown in liquid medium containing serum or N-acetylglucosamine revealed vigorous filamentation (Fig. 5A and 5B). In contrast, the *ndt80* mutants differentiated into short and swollen elongated cells with a high frequency of lateral branches in the presence of the serum (Fig. 5A).

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Similar results were obtained under hypoxic conditions and at pH 8. Indeed, while the wt and revertant strains showed abundant filamentation at the edge of colonies, *ndt80* developed smooth colonies with no mycelial growth even after prolonged incubation (Fig. 5C and 5D). This filamentation defect of *ndt80* mutants was also observed under other filament-inducing conditions, namely RPMI and spider media (data not shown).

CaNdt80p is required for the activation of hyphae specific genes

To gain insight into the role of CaNdt80p in mediating hyphal growth in *C. albicans*, we used microarrays to compare the transcriptomes of *ndt80* mutant and WT cells grown in the presence of serum at 37°C. Using a statistical significance analysis with an estimated false discovery rate of 5%, in addition to a stringent cut-off of 3-fold, we found that *ndt80* mutant cells have a severe gene expression defect as they failed to fully activate 82 and to repress 41 genes (Table S2). As illustrated in Figure 6, the *ndt80* mutant is unable to up regulate genes that have been previously characterized as being activated during the yeast to hyphae switch, including genes encoding cell wall components (*HWP1*, *ECE1, RBT4*, *ALS3*, *ALS10* and *HYR1*), a superoxide dismutase (*SOD5*) as well as two secreted aspartyl proteinases (*SAP4* and *SAP5*). Real time quantitative PCR was used to confirm the expression defect of some of these genes specifying the yeast-to-hyphae transition (Table 3).

Interestingly, activation of two TF required for the positive regulation of hyphae-specific genes, Ume6p and Tec1p, was found to be dependent on CaNdt80p. Additionally, *ndt80* mutants failed to downregulate genes repressed during hyphae formation, including

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YWP1, *CAX4*, *MNN22*, *RHD1*, *RHD3*, *ALD5* and the transcriptional repressor *NRG1* (Fig. 6). This clearly demonstrates that CaNdt80p is required for both the activation and the repression of genes characterizing the morphogenetic transcriptional program in *C. albicans*.

CaNdt80p is required for full virulence in a mouse model

The ability of *C. albicans* to switch from yeast to hyphae is critical for host invasion and virulence. Since *ndt80* mutants were unable to form hyphae in response to different filament-inducing conditions, we investigated if this TF is required for *C. albicans* virulence using a mouse model. The *C. albicans* strains DAY286 (wt), *ndt80* mutant strain, and a revertant were tested in a mouse model for systemic infection by intravenous injection in the tail vein. As shown in Figure 7A, while 70 % of the mice infected with WT and revertant strains became moribund within 12 days post-infection, none of *ndt80*-infected mice showed any clinical signs of advanced infection such as ruffled fur, hunch back or extreme lethargy until the end of the experiment (day 21). Fungal loads from kidney tissues were also examined. As shown in Figure 7B, fungal loads were significantly lower in *ndt80*-infected mice compared to mice challenged with the wt or revertant strains. Taken together these findings demonstrate that Ndt80p is a critical determinant of *C. albicans* pathogenicity.

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Discussion

CaNdt80p is a novel transcriptional regulator unique to the *Saccharomycotina* CTG clade

In the present study, we have shown that CaNdt80p, encoded by ORF19.2119, has a unique functional domain organization distinct from ScNdt80p. However, another member of Ndt80/PhoG TF family, encoded by orf19.513, exist in *C. albicans* and in addition to a significant sequence similarity, this TF showed exactly the same domain organization as the meiosis-specific TF ScNdt80p. This suggests that ORF19.513 the "true" *C. albicans* ortholog of ScNdt80p. Nevertheless, we have decided to continue using the common name *CaNDT80* to define ORF19.2119 as this gene has already been the subject of several publications by us and others and we have been unable to find any growth condition the would result in the transcription of the *ORF19.513* gene (results not shown).

The presence of a ScNdt80p-like TF in all ascomycetes suggests an important function of this regulator (Fig.1B). Surprisingly, ORF19.2119-like TFs were found exclusively in fungi belonging to the monophylic CTG clade of *Saccharomycotina*. In addition to *C. albicans*, this group contains a large number of closely related pathogenic yeasts such a *C. parapsilosis*, *C. tropicalis*, *C. lusitaniae* and *C. guilliermondii*. Recently, Butler et al. (3) revealed significant expansions of cell wall, secreted aspartases and transporter gene families in pathogenic species of the CTG clade, suggesting adaptations associated with pathogenesis. Such a large-scale amplification of gene family and its contributions to promoting virulence has also been attributed to other ascomycete and basidiomycete

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species (34, 37). Gene duplication and the expansion of multiple gene families are considered to be a major force in evolution by allowing functional innovation. These observations argue that the presence of two members of Ndt80/PhoG TF family in *C. albicans*, with highly similar DBD, is most likely the result of gene-duplication event from a common ancestor. Taken into consideration the role of CaNdt80p in virulence, the appearance or the retainment of this TF in *Candida* clade could be related to its critical role in the adaptation to its mammalian host environment.

CaNdt80p is a general transcriptional regulator acting as both an activator and a repressor

Using the global and unbiased approach of ChIP-Chip assays, we have demonstrated that CaNdt80p occupies a large number of promoter regions representing 23% of *C. albicans* genes (36). In addition, we have clearly demonstrated that CaNdt80p is required for the modulation of different biological processes, such as, cell separation, hyphal growth, virulence and azole sensitivity. Taken together, these results suggest that CaNdt80p is a multifunctional general transcriptional regulator. Gene expression analysis indicated that some direct targets of CaNdt80p were upregulated, whereas others were repressed in *ndt80* mutants. These findings suggest that this TF plays bifunctional roles as a repressor and an activator. Interestingly, in addition to structural and functional similarities between the CaNdt80p and p53 family members (20), p53 also has positive or negative effects on the activity of its target promoters (31).

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Intriguingly, CaNdt80p was found to bind a large number of gene promoters that did not show any significant changes in gene expression under yeast promoting conditions when this TF was absent. This phenomenon seems to be common in human TFs in which changing the level of a TF alters the expression level of only 1-10% of its known target genes (10). It seems therefore that only a small proportion of the binding sites for a factor might be functional in a given cell type and that their functionality could be determined by cell specific-partners that need to be recruited for transcriptional activation or repression. Alternatively, TF networks may be fairly robust and able to compensate for a missing regulator. In our study, this can be illustrated by the example of the promoter region of *NRG1*, which exhibits significant Ndt80p-binding in cells growing as yeasts. However, no *NRG1* transcript level alteration was observed in *ndt80* mutants grown under the same conditions. On the other hand, transcriptional profiling in the presence of serum revealed that the *ndt80* mutant failed to downregulate *NRG1* in response to serum. Assuming that Ndt80p binds to the *NRG1* promoter under hyphae promoting conditions, this suggests that the repression of this transcriptional repressor requires a hyphae-specific partner.

Ndt80p is a new regulator of cell separation in *C. albicans*

In *S. cerevisiae*, cell separation occurs during the G1 phase and is achieved by the degradation of septal components, composed essentially of chitin, which holds the mother and daughter cells together after cytokinesis (4,9). Degradation of septal materials is accomplished by the endo-chitinase Cts1p, which is responsible for the lysis of the primary septum at the neck (19). In addition to chitinase, glucanses such as Eng1p,

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Scw11p and Sun4p play a complementary role and are required for dissolution of the secondary septum and/or the surrounding cell wall materials holding the mother and daughter cells together (25, 42). In addition to the tight spatial regulation of hydrolytic enzymes, a strict temporal regulation during the cell cycle is also required (42). In both *S. cerevesiae* and fission yeast, this is achieved by the transcription factor Ace2p, which activates the expression of the chitinase *CTS1* and other glucanases specifically at the G1 phase (9). In *C. albicans*, deleting Ca*ACE2* results in a dramatic defect in cell separation as well as attenuated virulence (18). Additionally, it was shown that CaAce2p is required for the transcriptional activation of the chitinase Cht3p and other glucanases, highlighting an evolutionary conserved role of this TF in fungi (18, 26). Recent genome-wide investigations using DNA microarrays have revealed four successive waves of genes that are expressed periodically during the *C. albicans* cell cycle (7). Among these waves, *ACE2* was found to peak periodically during the G2/M transition and to activate cell separation genes (*CHT3*, *DSE1*, *SCW4*, *SCW11* and *ENG1*), which therefore are transcribed periodically at the M/G1 transition. Interrogating the Candida Cell Cycle database (www.bri.nrc.ca/candida/cycle/) for cycling transcripts revealed that *NDT80* was not considered as a periodic gene, however, a slight peak was observed exactly at the G2/M transition in synchrony with *ACE2*. Based on this observation, the cell separation role of Ndt80p might be cell cycle-regulated as it is for Ace2p.

In this work, we have reported a novel function of Ndt80p in controlling cell separation in *C. albicans* through direct transcriptional activation of genes encoding the endo-chitinase Cht3p and the β-glucanase Sun41p. It is therefore likely that cell separation

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completion in this pathogen is the result of contributions from both Ace2p and Ndt80p gene targets. While both Ndt80p and Ace2p are required for cell separation during mitotic exit, the overlap between the targets of these two TFs consists of only two genes, *CHT3* and *SUN41* (26). Ndt80p was not found to be required for transcriptional activation of other Ace2p cell separation-targets such as *DSE1*, *DSE4* and *SCW11*. Considering that Ndt80p was not found in the promoter region of *ACE2* (36) and is not required for its proper expression in addition to the fact that *NDT80* expression was not altered in *ace2* mutants, the two TFs might operate independently in two distinct transcriptional regulatory networks to control cell separation.

Ndt80p plays a central role in hyphal development and virulence in *C. albicans*

In *C. albicans*, the role of transcriptional regulators of hyphal growth has been the subject of numerous investigations. In this work we have enriched the repertoire of *C. albicans* filamentation-TFs by demonstrating the critical function of CaNdt80p in hyphal development. In *C. albicans*, the yeast-to-hyphae transition is triggered by various environmental stimuli, such as serum, neutral pH, high temperature, nutrient starvation and CO_2 (1). Deletion of Ca*NDT80* abolished the ability of *C. albicans* to undergo the yeast-to-hyphae transition in response to a large set of hyphae-inducing conditions. Since sensing of filamentation stimuli operates through a variety of distinct sensing/signalisation pathways, Ndt80p is thus thought to act as a critical downstream effector promoting hyphae formation in response to different signals conveyed by different upstream pathways.

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Transcriptional profiling revealed that the hyphal defect of *ndt80* mutants was correlated whith the inability to activate hyphae-specific genes such as *HWP1*, *ECE1, RBT4*, *ALS3*, *HYR1*, *SAP4* and *SAP5*. Additionally, given the fact that Ndt80p can act as a repressor, hyphal defect of the *ndt80* mutants could also be the consequence of the non-repression of yeast-specific genes such as *YWP1*, *CAX4*, *MNN22*, *RHD1*, *RHD3*, *ALD5*. Based on our transcriptional profiling data, the molecular basis of the hyphal defects of the *ndt80* mutants can be explained by three possibilities: i) *ndt80* cells fail to repress the transcriptional repressor Nrg1p. Taking into account the critical role of Nrg1p in hyphae-specific gene regulation (17) together with the occupancy of the *NRG1* promoter by Ndt80p (36), *C. albicans* filamentous growth mediated by CaNdt80p might be an outcome of the release of Nrg1p-repression at hyphae-specific gene promoters. ii) *ndt80* cells fail to activate the expression of genes encoding the transcriptional activators Ume6p and Tec1p. In our previous study, the *UME6* and *TEC1* promoters were shown to be bound by Ndt80p and we have shown here that *UME6* and *TEC1*-activation in the presence of serum require Ndt80p. Thus, CaNdt80p acts upstream of Ume6p and Tec1p, which, in turn, are implicated in activating the filamentation transcriptional program. iii) *ndt80* cells fail to activate key hyphal specific genes. In addition to the indirect regulation models we cannot rule out the possibility that CaNdt80p directly activates part of the hyphae transcriptional responses since it was found to bind the promoters of many hyphae-specific genes, such as, *ECE1*, *ALS3*, *ALS1*, *HWP1*, *HYR1* and *RBT4.*

The ability of *C. albicans* to undergo morphological switching is a critical pathogenicity determinant. In fact, hyphae differentiation facilitates the invasion of host tissues and also

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helps *C. albicans* to escape from phagocytosis (22, 35). The loss of virulence of *ndt80* mutants is most likely attributed to the key role of Ndt80p in controlling hyphal growth. However, the ability of this TF to activate other virulence related functions such as adhesion (*HWP1*, *ALS1*, *ALS3* and *ALS10)* (5, 32, 38) or extracellular proteolytic activity (*SAP4* and *SAP5*) (28) cannot be ruled out.

Acknowledgments

Thanks also to members of the BRI Microarray Lab and the BRI Animal Facility, especially, Khairul Islam, Jean-Sébastien Deneault, Mario Mercier, and Jessy Tremblay for technical assistance. This work was supported by grants from Canadian Institute of Health Research (CIHR) to A.N. (MOP-42516). C.A. was supported by an Alexander Graham Bell CGS-NSERC scholarship. This is NRC publication number 50681.

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Table 1. *Candida albicans* strains used in the study

Strain	Genotype	Source
BWP17	*his1/his1 ura3/ura3 arg4/arg4*	Wilson et al. (1999)
DAY286	*his1/his1 ura3/ura3 arg4/arg4::pARG4::URA3*	Davis et al. (2002)
AS31	*ndt80::HIS1/ndt80::URA3 arg4/arg4*	Sellam et al. (2009)
AS32	*ndt80::HIS1/ndt80::HIS1 arg4/arg4*	Sellam et al. (2009)
AS33	*ndt80::HIS1/ndt80::HIS1 RP10/rp10::pCIp10-NDT80 arg4/arg4*	Sellam et al. (2009)
AS40	*ndt80::HIS1/ndt80::HIS1 RP10/rp10::pCIpACT1-CHT3 arg4/arg4*	This study
AS41	*ndt80::HIS1/ndt80::HIS1 RP10/rp10::pCIpACT1-SUN41 arg4/arg4*	This study

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Table 2. Primers used in the study

Primer name	Primer sequence	Purpose
Cht3F1	CGACGCGTATGCTATACTTGTTAACTATATTTTC	CHT3 overexpression
Cht3R1	CTAGCTAGCATTATAGATAACCACTGTACTTGGT	
Sun41F1	ATGAGATTTTCACAAGCTACTGTT	SUN41 overexpression
Sun41R1	CTAGCTAGCATTATACAAGACAAAGTCAGCTTC	
qCht3F2	ACTACCTCCACAGCACCAAC	Quantitative real-time PCR
qCht3R2	GTAGAAGTGGCAGGTTTAGTTG	
qSun41F2	TGTGAATGGGGTGTCAAGAA	Quantitative real-time PCR
qSun41R2	AGCACCACCTCTCCAAGTGT	
qAct1 F1	GAAGCCCAATCCAAAAGA	Quantitative real-time PCR
qAct1 R1	CTTCTGGAGCAACTCTCAATTC	
qAls3 F1	CGGTTGCGACTGCAAAGAC	Quantitative real-time PCR
qAls3 R1	GACCAACCCAAAACAGCATTCC	
qHwp1 F1	CAGTTCCACTCATGCAACCATC	Quantitative real-time PCR
qHwp1 R1	GCAATACCAATAATAGCAGCACCG	
qYwp1 F1	CTG ATA TTC GTA ATG CTG GTA AAG TG	Quantitative real-time PCR
qYwp1 R1	GGA GTT TCA CCC ATT AAT CTT CTT C	
qEce1 F1	CCGGCATCTCTTTTAACTGG	Quantitative real-time PCR
qEce1 R1	GAGATGGCGTTCCAGATGTT	
qCax4 F1	TCAATTCATGGGATTTTTCG	Quantitative real-time PCR
qCax4 R1	CCCCGTAATTAATCCAGCAA	
qNrg1 F1	TGCAACCCCAACAAACACTA	Quantitative real-time PCR
qNrg1 R1	TGACGTTGTTGATATGATGCTG	
qTec1 F1	TGGTGCTTATTCACGTGTCC	Quantitative real-time PCR
qTec1 R1	GTGGTGGTCATGCCAATAGT	
qRBT4 F1	CGATGCTGATGGTGGTAATG	Quantitative real-time PCR
qRBT4 R1	TTGGTCATCTGAAGGGAAGC	

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Table 3. Quantitative real-time PCR analysis of genes identified as differentially expressed by microarray experiment in wt vs *ndt80* comparison under hyphae promoting conditions.

Gene	orf	Microarray[1]	RT qPCR[2]
ALS3	orf19.1816	0.03	5.08E-03 ± 0.0
HWP1	orf19.1321	0.08	8.73E-03 ± 0.0
ECE1	orf19.3374	0.03	2.16E-02± 0.2
YWP1	orf19.3618	30.59	867.06 ± 0.4
CAX4	orf19.3682	5.10	19.42 ± 3.2
NRG1	orf19.7150	3.87	7.16 ± 0.1
TEC1	orf19.5908	0.20	0.23 ± 0.1
RBT4	orf19.6202	0.02	0.34 ± 0.1

[1]Average fold change

[2]Each value is the mean ± standard deviation of two independent experiments each with three replicates.

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Figure 1. NDT80/PhoG transcription factor family across *Ascomycota*
A. Functional domain organization of NDT80/PhoG family members in *Saccharomyces cerevesiae* and *Candida albicans*. Both the DNA-Binding Domain (Ndt80 DBD) and putative glutamine-rich Activation Domain (Ndt80p AD) are represented.

B. Structural organization of NDT80/PhoG family members across different classes of ascomycetes. The topology of the tree was based on (12). The CTG clade is highlighted by a red rectangle. WGD identifies the clade containing species in which whole-genome duplication has occurred.

Figure 2. Ndt80p is required for cell separation completion.
A. Images of wt (DAY286), *ndt80* mutant (AS31) and revertant (AS33) strains showing the cell separation defect. Cells were stained with calcofluor white.
B. Quantification of cell separation defect. Percentage of chains of cells containing 1–2, 3–4, 5–6 or more than 6 cells.

Figure 3. Ndt80p directly regulates genes implicated in cell separation
A. Relationship between Ndt80p bound genes and genes showing altered expression in *ndt80* mutant (AS31).
B. Ndt80p promoter occupancies of the endo-chitinase Cht3p and the glucosidase Sun41p as determined in Sellam et al. (2009). The positions of MSE motifs are shown.

Figure 4. *ndt80* cell separation defect is reverted by the overexpression of *CHT3* or *SUN41*.
A. Average transcript levels of *CHT3* and *SUN41* in overexpression mutants relative to the wt stain (DAY286). For each genes two clones (Clone C1 and C20 for *ndt80*/*Act*::SUN41 strain and clone C1 and C11 for *ndt80*/Act::CHT3 strain) were evaluated. The reported values are the means of two independent experiments.
B. Evaluation of the percentage of chains of cells with 1–2, 3–4, 5–6 or more than 6 cells in overexpressing strains.

Figure 5. CaNdt80p is essential for hyphal growth in response to different filament-inducing conditions
Cell morphology of wt (DAY286), *ndt80* mutant (AS31) and revertant (AS33) strains on liquid media supplemented with fetal bovine serum (**A**) or N-acetyl-D-glucosamine (**B**). Colony morphology of wt, *ndt80* and revertant strains after five days growth on solid media under hypoxic condition (**C**) or in M199 medium at pH 8 (**D**).

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Figure 6. CaNdt80p is required for the activation of hyphae-specific genes
Overlap between genes differentially regulated during yeast-to-hyphae switch as determined by (29) and (17), and genes down- (**A**) or upregulated (**B**) in a wt vs *ndt80* comparison.

Figure 7. Ndt80p is required for full virulence in B6 mouse model.
A. Survival of mice infected with *ndt80* mutant, *ndt80* revertant, and wt parental strains. Mice were inoculated by tail vein injection, and survival was measured over a 21-d period. The kidney fungal load was determined 7 days after injection (**B**).

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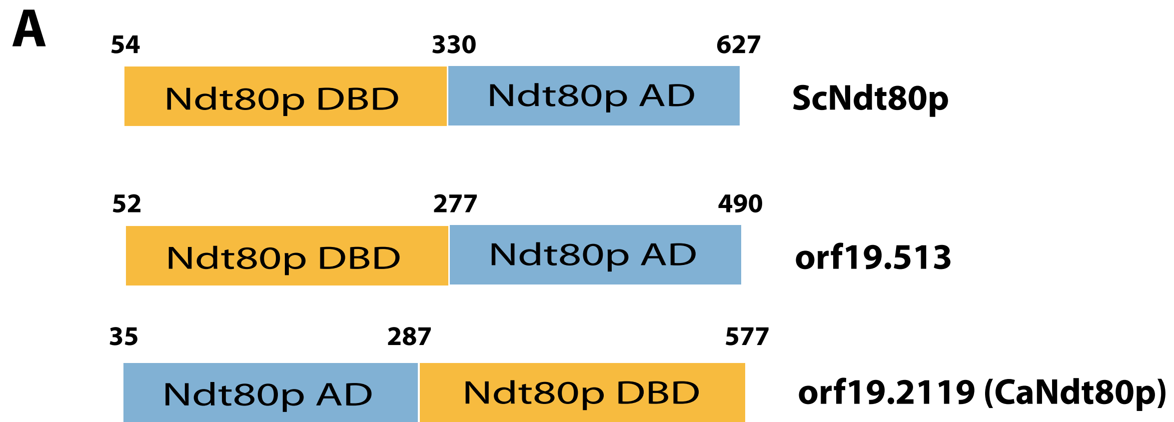

B

S. pombe
Ndt80p DBD
Ndt80p AD
Archaeascomycetes
Y. lipolytica
C. lusitaniae
C. guilliermondii
D. hansenii
Ndt80p AD
Ndt80p DBD
CTG clade
C. tropicalis
C. albicans
C. dubliniensis
Ndt80p DBD
Ndt80p AD
C. parapsilosis
L. elongisporus
A. gossypii
S. castellii
WGD
S. bayanus
S. kudriavzevii
Ndt80p DBD
Ndt80p AD
S. mikatae
S. paradoxus
S. cerevesiae
S. kluyveri
K. waltii
Hemiascomycetes
A. nidulans
Ndt80p DBD
Ndt80p AD
M. grisea
N. crassa
Euascomycetes

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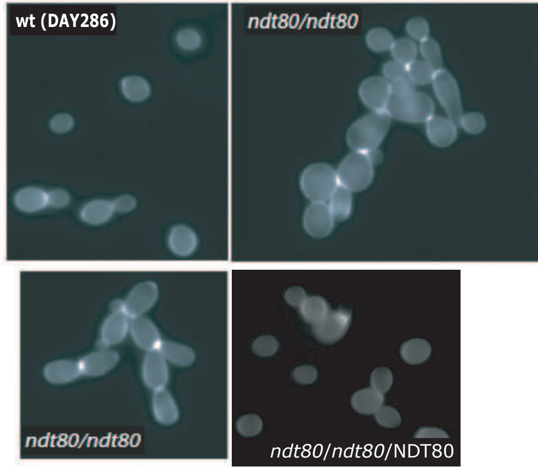

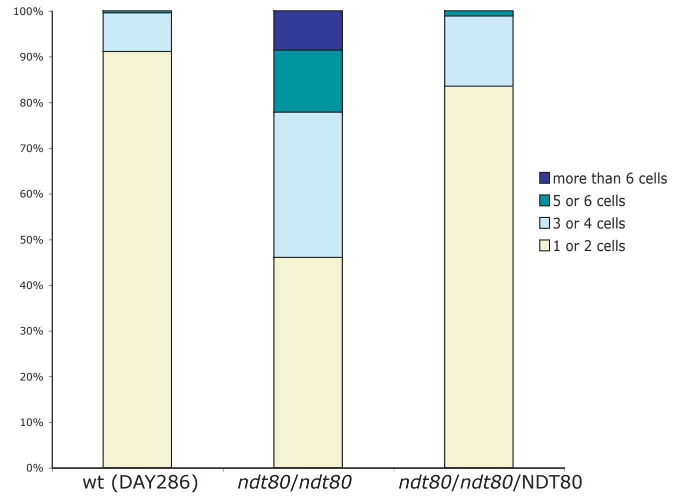

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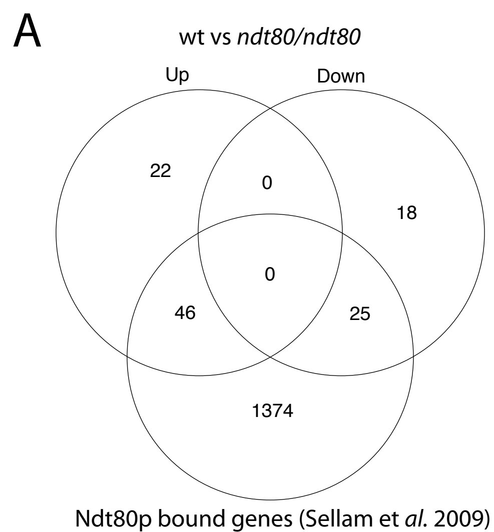

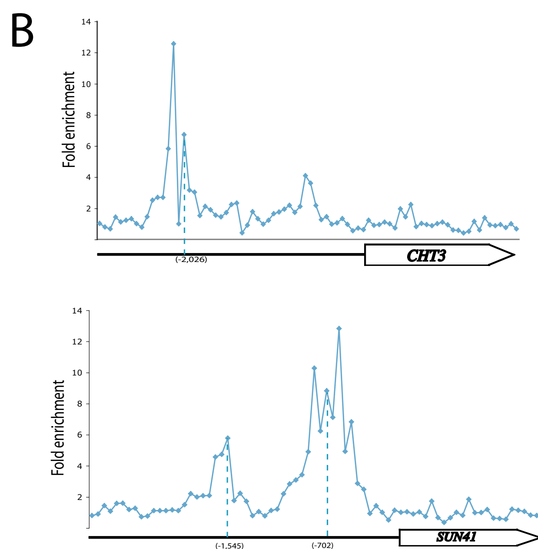

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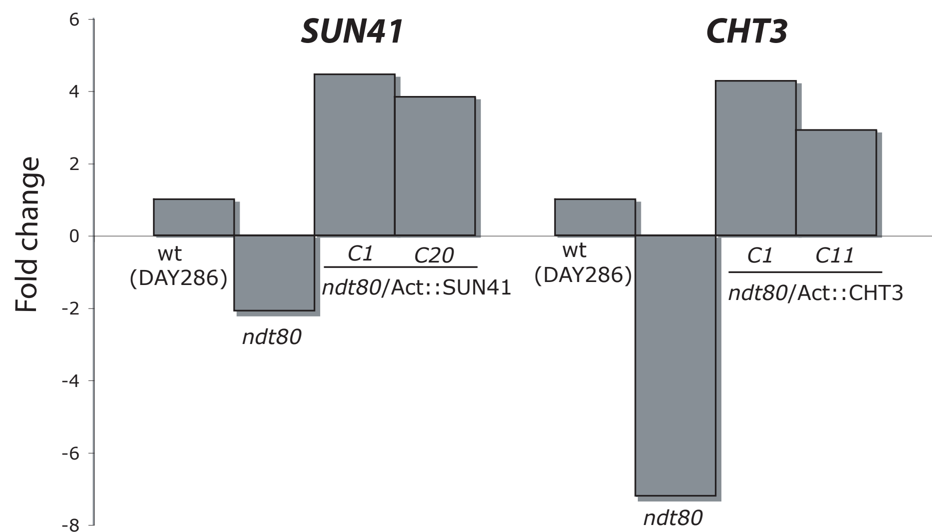
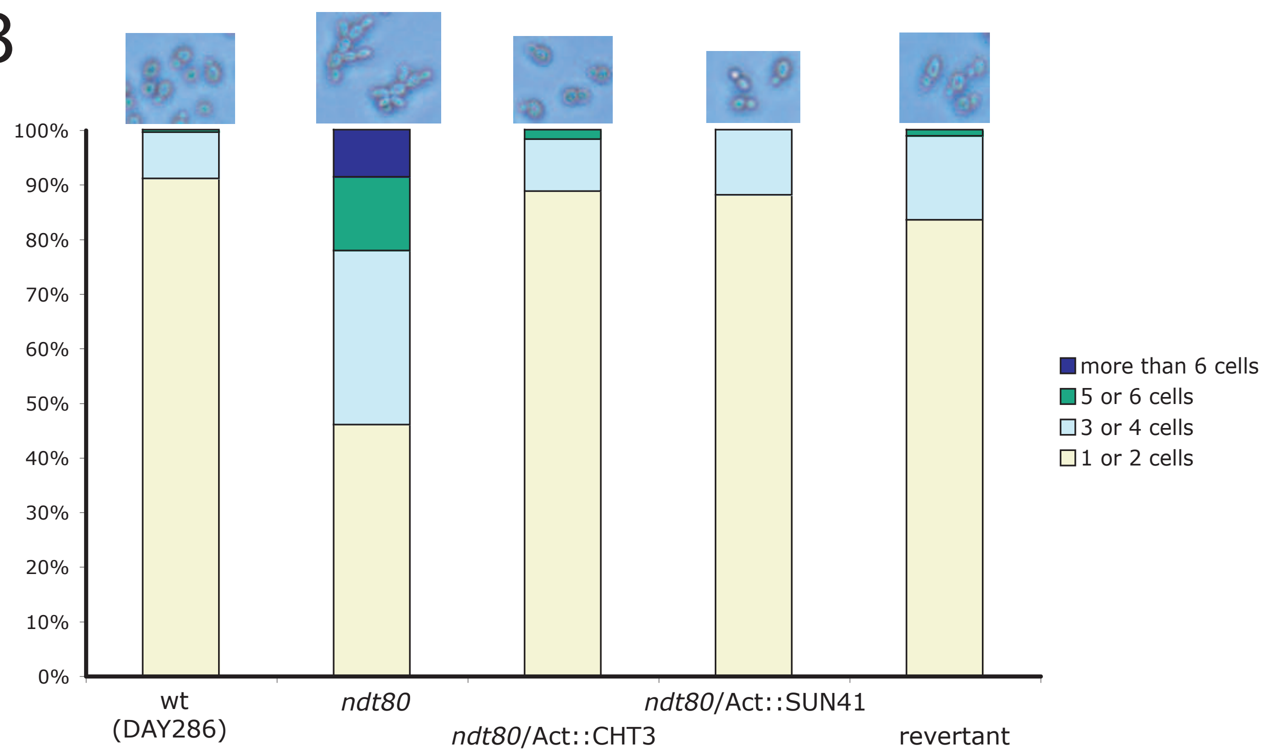
A



B



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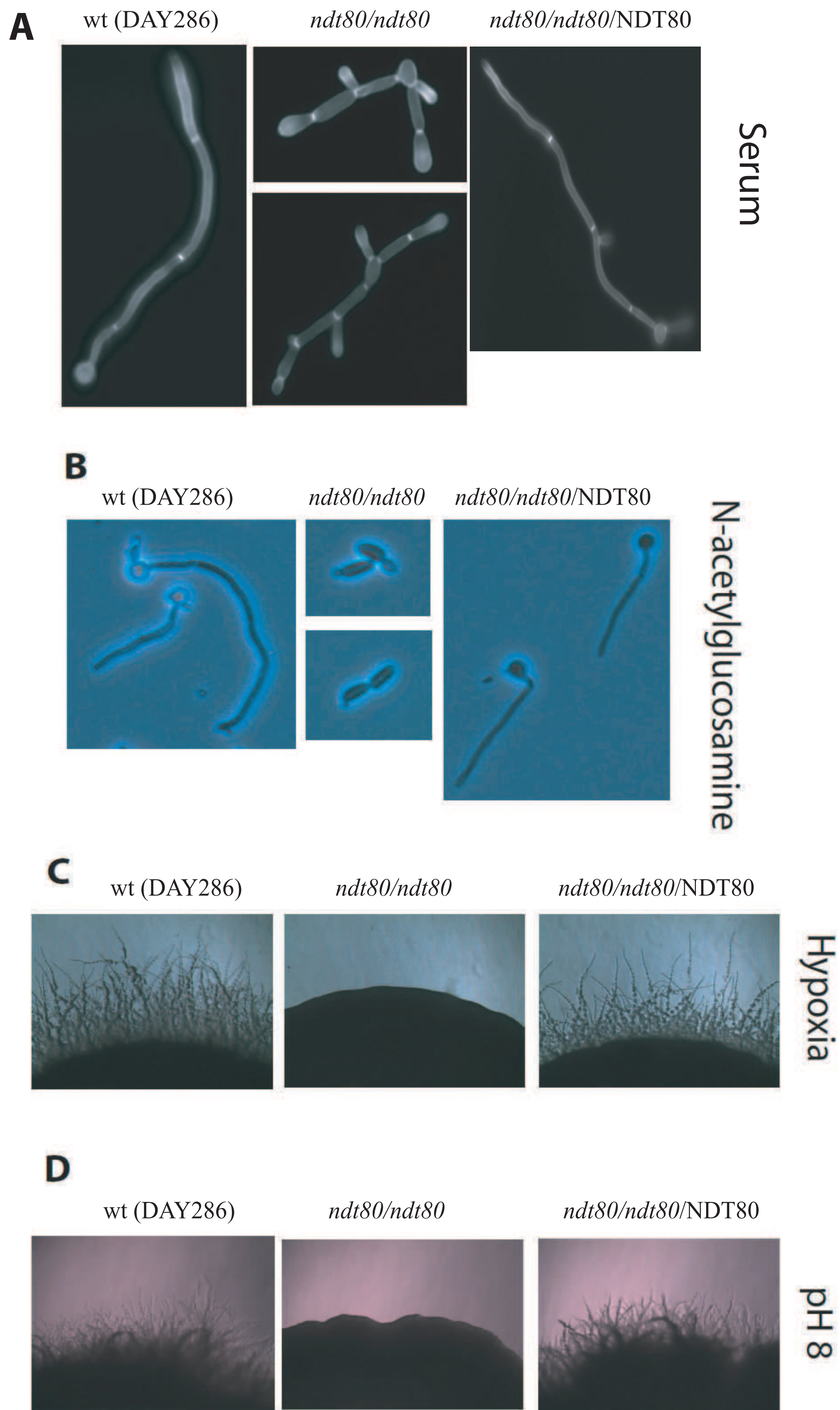

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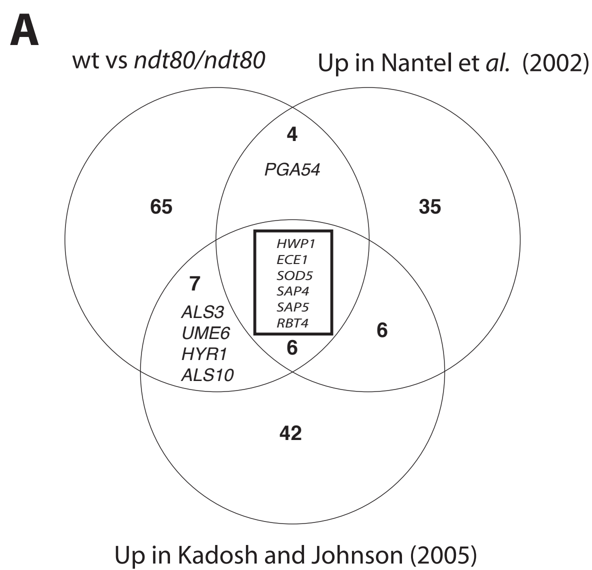

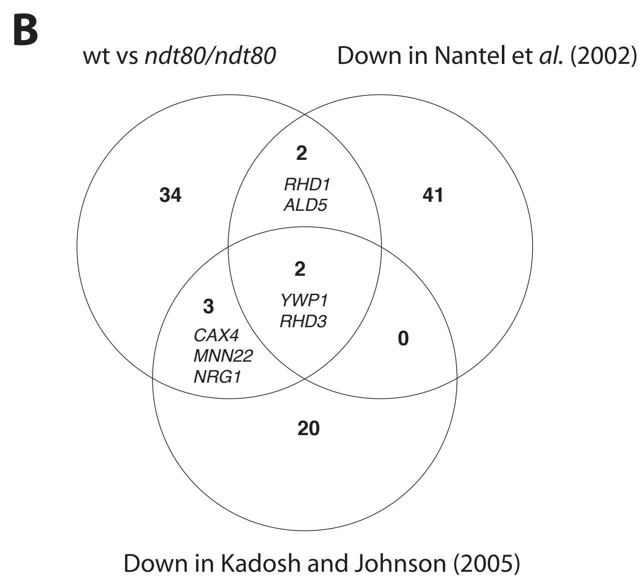

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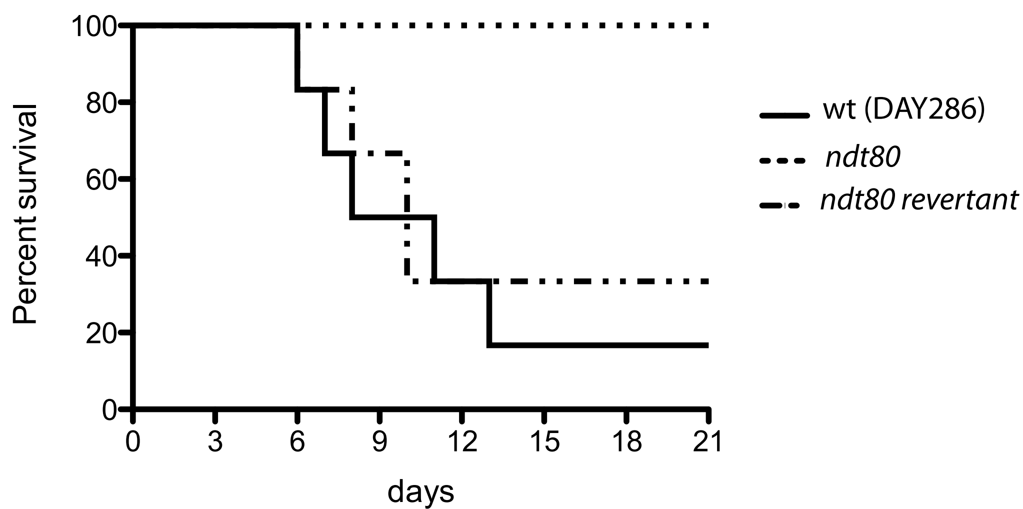

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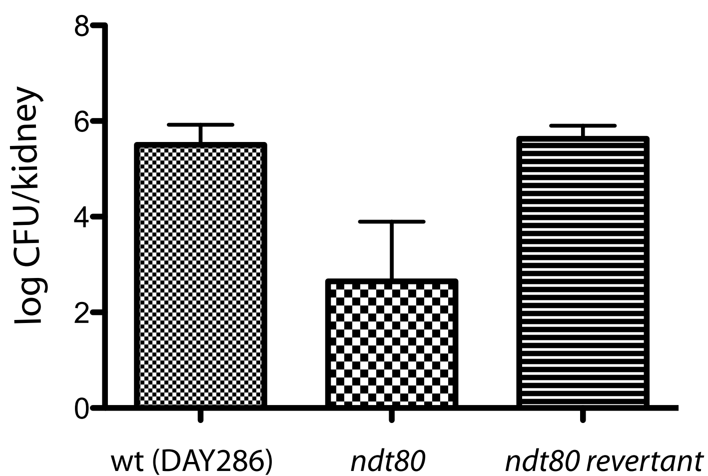

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AS31	*ndt80::HIS1/ndt80::URA3 arg4/arg4*	Sellam et al. (2009)
AS32	*ndt80::HIS1/ndt80::HIS1 arg4/arg4*	Sellam et al. (2009)
AS33	*ndt80::HIS1/ndt80::HIS1 RP10/rp10::pCIp10-NDT80 arg4/arg4*	Sellam et al. (2009)
AS40	*ndt80::HIS1/ndt80::HIS1 RP10/rp10::pCIpACT1-CHT3 arg4/arg4*	This study
AS41	*ndt80::HIS1/ndt80::HIS1 RP10/rp10::pCIpACT1-SUN41 arg4/arg4*	This study

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Table 2. Primers used in the study

Primer name	Primer sequence	Purpose
Cht3F1	CGACGCGTATGCTATACTTGTTAACTATATTTTC	CHT3 overexpression
Cht3R1	CTAGCTAGCATTATAGATAACCACTGTACTTGGT	
Sun41F1	ATGAGATTTTCACAAGCTACTGTT	SUN41 overexpression
Sun41R1	CTAGCTAGCATTATACAAGACAAAGTCAGCTTC	
qCht3F2	ACTACCTCCACAGCACCAAC	Quantitative real-time PCR
qCht3R2	GTAGAAGTGGCAGGTTTAGTTG	
qSun41F2	TGTGAATGGGGTGTCAAGAA	Quantitative real-time PCR
qSun41R2	AGCACCACCTCTCCAAGTGT	
qAct1 F1	GAAGCCCAATCCAAAAGA	Quantitative real-time PCR
qAct1 R1	CTTCTGGAGCAACTCTCAATTC	
qAls3 F1	CGGTTGCGACTGCAAAGAC	Quantitative real-time PCR
qAls3 R1	GACCAACCCAAAACAGCATTCC	
qHwp1 F1	CAGTTCCACTCATGCAACCATC	Quantitative real-time PCR
qHwp1 R1	GCAATACCAATAATAGCAGCACCG	
qYwp1 F1	CTG ATA TTC GTA ATG CTG GTA AAG TG	Quantitative real-time PCR
qYwp1 R1	GGA GTT TCA CCC ATT AAT CTT CTT C	
qEce1 F1	CCGGCATCTCTTTTAACTGG	Quantitative real-time PCR
qEce1 R1	GAGATGGCGTTCCAGATGTT	
qCax4 F1	TCAATTCATGGGATTTTTCG	Quantitative real-time PCR
qCax4 R1	CCCCGTAATTAATCCAGCAA	
qNrg1 F1	TGCAACCCCAACAAACACTA	Quantitative real-time PCR
qNrg1 R1	TGACGTTGTTGATATGATGCTG	
qTec1 F1	TGGTGCTTATTCACGTGTCC	Quantitative real-time PCR
qTec1 R1	GTGGTGGTCATGCCAATAGT	
qRBT4 F1	CGATGCTGATGGTGGTAATG	Quantitative real-time PCR
qRBT4 R1	TTGGTCATCTGAAGGGAAGC	

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Table 3. Quantitative real-time PCR analysis of genes identified as differentially expressed by microarray experiment in wt vs *ndt80* comparison under hyphae promoting conditions.

Gene	orf	Microarray[1]	RT qPCR[2]
ALS3	orf19.1816	0.03	5.08E-03 ± 0.0
HWP1	orf19.1321	0.08	8.73E-03 ± 0.0
ECE1	orf19.3374	0.03	2.16E-02± 0.2
YWP1	orf19.3618	30.59	867.06 ± 0.4
CAX4	orf19.3682	5.10	19.42 ± 3.2
NRG1	orf19.7150	3.87	7.16 ± 0.1
TEC1	orf19.5908	0.20	0.23 ± 0.1
RBT4	orf19.6202	0.02	0.34 ± 0.1

[1]Average fold change

[2]Each value is the mean ± standard deviation of two independent experiments each with three replicates.

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