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Dur3 is the major urea transporter in *Candida albicans* and is co-regulated with the urea amidolyase Dur1,2

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Hemiascomycetes, including the pathogen *Candida albicans*, acquire nitrogen from urea using the urea amidolyase Dur1,2, whereas all other higher fungi use primarily the nickel-containing urease. Urea metabolism via Dur1,2 is important for resistance to innate host immunity in *C. albicans* infections. To further characterize urea metabolism in *C. albicans* we examined the function of seven putative urea transporters. Gene disruption established that Dur3, encoded by orf 19.781, is the predominant transporter. [^{14}C]Urea uptake was energy-dependent and decreased approximately sevenfold in a *dur3*Δ mutant. *DUR1,2* and *DUR3* expression was strongly induced by urea, whereas the other putative transporter genes were induced less than twofold. Immediate induction of *DUR3* by urea was independent of its metabolism via Dur1,2, but further slow induction of *DUR3* required the Dur1,2 pathway. We investigated the role of the GATA transcription factors Gat1 and Gln3 in *DUR1,2* and *DUR3* expression. Urea induction of *DUR1,2* was reduced in a *gat1*Δ mutant, strongly reduced in a *gln3*Δ mutant, and abolished in a *gat1*Δ *gln3*Δ double mutant. In contrast, *DUR3* induction by urea was preserved in both single mutants but reduced in the double mutant, suggesting that additional signalling mechanisms regulate *DUR3* expression. These results establish Dur3 as the major urea transporter in *C. albicans* and provide additional insights into the control of urea utilization by this pathogen.

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INTRODUCTION

Nitrogen sources play important roles in regulating fungal dimorphism. For example, *Ceratocystis ulmi* cells grow as yeasts in the presence of proline but as hyphae in the presence of ammonia, arginine and most other nitrogen sources (Kulkarni & Nickerson, 1981). *Trigonopsis variabilis* cells grow as budding yeasts in the presence of ammonium sulfate and as triangles with methionine (Sentheshanmuganathan & Nickerson, 1962).

Urea is an important nitrogen source for many fungi and is also important for microbial pathogenesis in humans. Urease is a virulence factor for some pathogenic fungi, including *Cryptococcus neoformans* (Cox *et al.*, 2000a) and *Coccidioides immitis* (Cole, 1997), and for the bacterial pathogens *Helicobacter pylori* (Eaton *et al.*, 1991) and *Proteus mirabilis* (Jones *et al.*, 1990).

Abbreviations: NCR, nitrogen catabolite repression; qPCR, quantitative PCR.

The role of urea in the dimorphic human fungal pathogen *Candida albicans*, however, has been unclear. Although Dastidar and co-workers reported in 1967 that many strains of *C. albicans* grow well with urea as the sole source of nitrogen (Dastidar *et al.*, 1967), this observation drew little attention based on numerous reports that *C. albicans* lacks urease (Odds, 1988). This apparent contradiction was recently explained by the discovery that *C. albicans* uses urea amidolyase to hydrolyse urea (Ghosh *et al.*, 2009). The enzyme urea amidolyase, encoded by *DUR1,2*, was first characterized in *Candida utilis* (Roon *et al.*, 1972). Dur1,2 is a multifunctional biotin-dependent enzyme (Roon & Levenberg, 1972) with domains for urea carboxylase and allophanate (urea carboxylate) hydrolase activity (Cooper *et al.*, 1980). Recently, we showed that higher fungi exhibit a dichotomy with regard to urea utilization (Navarathna *et al.*, 2010). All of the hemiascomycetes use urea amidolyase (Dur1,2). Most other higher fungi use urease exclusively, except the Sordariomycetes (*Magnaportha*, *Fusarium* and *Nectria*), which have both urease and urea amidolyase. Still,

the vast majority of yeasts/hemiascomycetes preserve the dichotomy by having urea amidolyase but not urease.

Why do *Candida* and *Saccharomyces* use an energy-dependent, biotin-containing urea amidolyase system when the same overall reaction could be accomplished by the simpler urease? This question becomes even more germane when we consider that all strains of *C. albicans* are biotin auxotrophs (Odds, 1988), and that two to four times as much biotin is required for maximum growth of *Saccharomyces cerevisiae* on urea, allantoic acid or allantoin as sole nitrogen source (Di Carlo *et al.*, 1953). We have suggested that urea amidolyase allows the hemiascomycetes to jettison their sole Ni(II)- and Co(II)-dependent enzyme and thus to require two fewer transition metals (Navarathna *et al.*, 2010). In addition, urea amidolyase may create a urea-dependent signalling pathway, and we have hypothesized that such a pathway might be related to fungal pathogenicity (Ghosh *et al.*, 2009).

While examining the role of arginine-induced germ tube formation in the escape of *C. albicans* from murine macrophages (Ghosh *et al.*, 2009), we deleted the *DUR1,2* gene from the wild-type strain A72. The *dur1,2*Δ mutant KWN6 was unable to grow on urea as the sole nitrogen source, stimulate germ tube formation in response to L-arginine or urea, or escape from the murine macrophage cell line RAW 264.7. These abilities were restored in the reconstituted strains KWN7 and KWN8 (Ghosh *et al.*, 2009).

Given the divergence in mechanisms for urea degradation, we examined whether a similar dichotomy exists in the mechanisms for urea transport. Here, we define the major urea transporter in *C. albicans*, and examine the regulation of urea transport and urea utilization by other known nitrogen sources and two transcription factors involved in the induction of genes that are subject to nitrogen catabolite repression (NCR).

METHODS

Strains and growth conditions. The *C. albicans* strains used for this study are listed in Table 1. For all experiments, *C. albicans* cells were grown overnight in 50 ml yeast peptone dextrose (YPD) medium at 30 °C with aeration, as previously described (Navarathna *et al.*, 2005). Cells were harvested by centrifugation at 5000 r.p.m. for 10 min, washed once with 50 ml sterile, non-pyrogenic normal saline (Quality Biological, Inc.) and resuspended in 10 ml saline before quantifying cell numbers using a Petroff–Hausser counting chamber. For gene expression analysis, *C. albicans* cells were grown for 16 h in 50 ml YPD medium (Kulkarni & Nickerson, 1981), washed three times with PBS, and added to glucose peptone (GP) medium containing 10 mM of the indicated nitrogen sources. Fresh cultures were started at OD_{600} 0.2 using overnight YPD cultures, and grown up to OD_{600} 0.8 (for about 4–5 h) for RNA extraction.

Disruption and complementation of *DUR3* (orf 19.781). A *DUR3* deletion cassette was constructed by amplifying the *DUR3* upstream and downstream regions from genomic DNA of strain SC5314 with the primer pairs DUR31/DUR32 and DUR33/DUR34, respectively (Table 2), and cloning the *Sac*I/*Sac*II- and *Xho*I/*Apa*I-digested PCR products on both sides of the *SAT1* flipper cassette of plasmid pSFS1, in which expression of the *caFLP* gene is controlled by the *SAP2* promoter (Reuß *et al.*, 2004). The *Sac*I–*Apa*I fragment from the resulting plasmid pDUR3M3 was used to sequentially delete the *DUR3* alleles of strain SC5314 by the *SAT1* flipping strategy, as described previously (Dunkel *et al.*, 2008), resulting in the homozygous *dur3*Δ mutants DUR3M4A and DUR3M4B. For reintroduction of a functional *DUR3* copy, the *DUR3* coding region plus 0.3 kb of upstream and 0.5 kb of downstream sequence was amplified with the primers DUR31 and DUR35, and the *Sac*I/*Sac*II-digested PCR product was substituted for the *DUR3* upstream fragment in pDUR3M3. The *Sac*I–*Apa*I fragment from the resulting plasmid pDUR3K1 was used to reintegrate *DUR3* at the endogenous locus of the *dur3*Δ mutants DUR3M4A and DUR3M4B, followed by recycling of the *SAT1* flipper cassette, to generate the complemented strains DUR3K2A and DUR3K2B.

Mouse candidiasis model. Outbred 6–8-week-old (18–20 g) BALB/c female mice obtained from Charles River Laboratories were randomly allocated to groups of five animals and housed and cared for according to guidelines of the National Cancer Institute (NCI) Animal Care and Use Committee. Each group of mice was inoculated intravenously in the lateral caudal tail vein using a 27-gauge needle with a volume of 0.1 ml containing 10^6 *C. albicans* cells (Navarathna *et al.*, 2005, 2007). Clinical signs of illness in each mouse were evaluated three times daily, and moribund mice that displayed sunken eyes, arched back posture, dehydration, ruffled hair or difficulty walking were euthanized immediately by placing them in a closed chamber filled with CO_2, and processed for complete necropsy and collection of tissues for histopathological examination.

RNA extraction and gene expression analysis by RT-PCR. RNA isolation was done using a standard hot phenol procedure (Köhrer & Domdey, 1991). Reverse transcription was conducted using 5 μg total RNA extracted from each sample using SuperScript III Reverse Transcriptase (Invitrogen) according to the manufacturer's instructions for oligo-dT priming. Quantitative PCR (qPCR) was conducted as previously described (Navarathna & Roberts, 2010) using ABsolute QPCR SYBR Green Mix (Thermo Scientific), an Opticon I instrument and Opticon I software (Bio-Rad). Samples were analysed by PCR in triplicate and normalized to internal CDC36 mRNA levels. Melting curve analysis was performed to ensure that a single product was produced in each reaction (Navarathna & Roberts, 2010). The qPCR primers used in this study are listed in Table 2, and all products were 75–125 bases in length.

Uptake of [^{14}C]urea. All *C. albicans* cells were grown overnight in 50 ml YPD, washed and resuspended in PBS, and used as the inoculum (OD_{600} 0.2) for fresh cultures in glucose phosphate proline (GPP) medium grown at 30 °C for 3–4 h while shaking at 150 r.p.m. Cell numbers were counted and adjusted so that all uptake assays employed an equal number of cells. Assays were done in GP buffer in 14 ml round-bottomed tubes (BD) with or without 5 mM sodium azide, using ~10^7 cells ml^{-1} and 1 μCi (3.7×10^4 Bq, 1.15 μg ml^{-1}, 19.2 μM) [^{14}C]urea (American Radiolabelled Chemicals). After incubation with orbital shaking at 150 r.p.m. for 5 min, unless otherwise indicated, triplicate 0.5 ml samples were collected. Two 0.2 ml volumes of the cell suspension were sequentially overlaid onto 100 μl 30 % sucrose in 5 × 49 mm microfuge tubes and centrifuged in a Beckman Microfuge B for 30 s. After removing the upper aqueous layer, the resulting cell pellet containing 4×10^6 cells was washed by adding 300 μl PBS and recentrifuged. After removing the supernatant fluid, the tips of the microfuge tubes containing the cell pellets were cut off and shaken in scintillation fluid before quantifying the internalized urea using a scintillation counter.

Table 1. Strains used in this study

Strain	Relevant genotype	Reference or source
SC5314	Wild-type	Gillum *et al.* (1984)
A72	Wild-type	ATCC* MYA-2430
KWN6	*dur1,2Δ/dur1,2Δ*	Ghosh *et al.* (2009)
KWN7	*dur1,2Δ/dur1,2Δ::DUR1,2*	Ghosh *et al.* (2009)
KWN8	*dur1,2Δ::DUR1,2/dur1,2Δ::DUR1,2*	Ghosh *et al.* (2009)
DUR3M4A and -B	*dur3Δ/dur3Δ*	This study
DUR3K2A and -B	*dur3Δ/dur3Δ::DUR3*	This study
GAT1M4A	*gat1Δ/gat1Δ*	Dabas & Morschhäuser (2007)
GLN3M4A	*gln3Δ/gln3Δ*	Dabas & Morschhäuser (2007)
Δ*gln3*GAT1M4A	*gat1Δ/gat1Δ gln3Δ/gln3Δ*	Dabas & Morschhäuser (2007)

*American Type Culture Collection.

Bioinformatics analysis. Sequences of fungal proteins were obtained from the National Center for Biotechnology Information (NCBI) (http://www.ncbi.nlm.nih.gov) and the Fungal Genome Initiative (http://www.broad.mit.edu/annotation/fgi/). *DUR3* homologues were identified using *S. cerevisiae* Dur3 (YHL016C) as a query for BLASTP searches. All BLAST searches were conducted using default parameters. We examined the distribution of *DUR3* in the genomes reported previously (Navarathna *et al.*, 2010).

Statistics. The probability of survival as a function of time was determined by the Kaplan–Meier method, and significance was determined by the log-rank (Mantel–Cox) test and the Jehan–Breslow–Wilcoxon test using GraphPad Prism software. qRT-PCR data were analysed using the Pfaffl method (Pfaffl, 2001) and were normalized in two steps. CDC36, an abundantly expressed nuclear protein (Collart & Struhl, 1994), was used as the internal control because we found its mRNA to be the most invariant for normalizing *C. albicans* gene expression analysis (Navarathna & Roberts, 2010; Pendrak *et al.*, 2004a, b, c). First, mean values for the internal CDC36 control were subtracted from the corresponding experimental sample, and the variance was calculated as the square root of the sum of the squares of the standard deviations. Second, each sample group was normalized to *C. albicans* grown in YPD medium, except in Fig. 2, where GPP was used. The results are

Table 2. Oligonucleotides used in this study

All primers are in 5′→3′ orientation. Introduced restriction sites are underlined.

Primer	Sequence
DUR31	TTGAGCTCGATAAGACTAAATGACTGCG
DUR32	CACTCCGCGGTAATGGGACATATGAATCAGCC
DUR33	CATTGACGATGCACTCGAGGAGAAGAAAGG
DUR34	GTCAGCATAATCAGGGCCCAATCAGAAGCC
DUR35	TAATCCGCGGTATTCTCATGATTTGCCCC
DUR1,2 RT LT	TTTGTTGGTCCATCTGGTGA
DUR1,2 RT RT	TCTGGCTTCTTTGGCATCTT
DUR RT LT	AGCTTGGCAAAATGAACACC
DUR RT RT	GCTGGGGTGACATATCCATC
DUR3 RT LT	ACAGTGCTCGAGAAGGTGGT
DUR3 RT RT	AAAGCAGCTGAAGCCAATGT
DUR4 RT LT	ATCCATGGGCTGGTTATTCA
DUR4 RT RT	CACCTGCCTGGTTCAAATCT
DUR7 RT LT	GGGTTATTTGGCCAATGTCT
DUR7 RT RT	AAAAGGGGACCAATCCAATC
NPR2 RT LT	AATGGCGGAAGAATGTCAAG
NPR2 RT RT	ACTTCCAGGGTAGGGCAAGT
5915 RT LT	ACGTTGACTCCGATCGAAAC
5915 RT RT	GATTGCAGCACCAGTAGCAA
5017 RT LT	TTTGTCCAGGTGTGATTCCA
5017 RT RT	GTGATATGCCGTCGAAATCC
CDC36 RT LT	GAGCGTCCAGTATAAATCCACCAC
CDC36 RT RT	TCAAGACGGGCTCCACATTACTAT

presented as fold change using 2^{-Ct} of this normalized C_t value. Statistical differences among groups were analysed with two-way analysis of variance (ANOVA) using GraphPad Prism software.

RESULTS

Expression of potential urea transporters

The yeast *S. cerevisiae* metabolizes urea by means of Dur1,2 and the urea transporters Dur3 and Dur4 (Cooper, 1982). *DUR1,2* and *DUR3* are inducible by allophanate, whereas *DUR4* encodes a constitutive urea-facilitated diffusion transporter (Cooper, 1982). The *C. albicans* orthologue *DUR1,2* was identified by Gene Ontology (GO) annotation. Remarkably, the *C. albicans* genome contains seven genes encoding putative urea transporters: *DUR3* (orf 19.781), *DUR3* (orf 19.6656), *DUR4* (orf 19.5677), *DUR7* (orf 19.7205), *NPR2* (orf 19.328), orf 19.5915 and orf 19.5017. The gene name *DUR3* has been used for both orf 19.781 and orf 19.6656, but orf 19.781 is the orthologue of *DUR3* of *S. cerevisiae,* and we therefore refer to this gene as *DUR3*. *DUR3*, *DUR4* and *DUR7* are fungal-specific in that they lack human or murine orthologues.

Based on gene expression levels in minimal medium, we separated the putative urea transporters into two categories. *DUR3* and orf 19.6656 reproducibly showed high expression, with C_t values less than 22, whereas the others all had C_t values less than 24. Addition of urea instead of ammonia to the growth medium (glucose phosphate urea; GPU) resulted in a 100-fold increase in *DUR3* gene expression but no increase for orf 19.6656 relative to the basal expression in YPD (Fig. 1a). None of the other five putative transporter genes showed significant responses to urea relative to that in YPD (Fig. 1b).

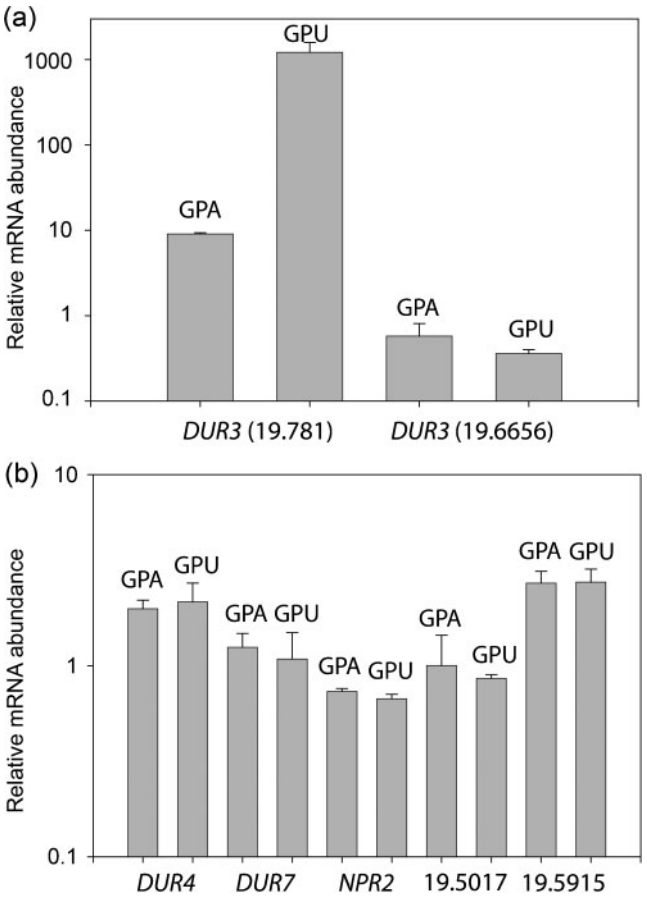

Fig. 1. Effect of urea on the expression of seven putative urea transporters. mRNA abundance was determined by qPCR using *C. albicans* A72 cells grown in GPA (glucose phosphate ammonia) or GPU medium. Experiments were performed in triplicate; error bars, SEM. Genes exhibiting higher expression are shown in (a) and low-expression genes are shown in (b). GPA and GPU expression levels were normalized to those of YPD. Cells grown in GPP medium showed induction of *DUR3* similar to that of cells grown in GPA (data not shown).

Urea induction of *DUR3* is not dependent upon *DUR1,2* expression

The *dur1,2*Δ mutant KWN6 and the complemented strain KWN8 (Ghosh *et al.*, 2009) were used to determine whether the *DUR3* urea response depended on a metabolite downstream of Dur1,2, such as allophanate. Expression of *DUR1,2* and *DUR3* was increased six- and 10-fold, respectively, for *C. albicans* A72 grown with urea (glucose phosphate proline urea; GPPU) rather than without (GPP; Fig. 2a, b). Expression and induction of *DUR1,2* (Fig. 2a) was lost in the *dur1,2*Δ mutant, and restored in the fully reconstituted strain KWN8 (Fig. 2a). However, in the *dur1,2*Δ mutant, *DUR3* expression was still increased ~20-fold by urea (GPPU) relative to the same strain in GPP. Thus, *DUR3* basal expression was slightly reduced in the absence of *DUR1,2*, but its relative induction by urea was preserved.

However, examining the time dependence of *DUR3* induction by urea in A72 (wild-type) versus KWN6 (*dur1,2*Δ) cells revealed a delayed effect of urea metabolism via Dur1,2 on *DUR3* induction (Fig. 2c). For this experiment, overnight *C. albicans* cultures grown in YPD were washed three times before transfer to GPU medium to examine gene expression induced by urea. Within 1 min of addition of urea, mRNA levels increased 400- to 600-fold in both cultures. This immediate induction was largely independent of Dur1,2. However, *DUR3* mRNA increased an additional threefold by 15 min in wild-type cells, whereas no further increase was seen in the mutant lacking *DUR1,2* (Fig. 2c). Therefore, a metabolite of urea downstream of Dur1,2 may account for a secondary induction of *DUR3*. Alternatively, upstream feedback inhibition of further *DUR3* induction may occur due to urea accumulation in the cell.

We also tested *DUR1,2* and *DUR3* expression in the presence of 10 mM allantoin. Allantoin, an intermediate of purine degradation that is subsequently converted to urea,

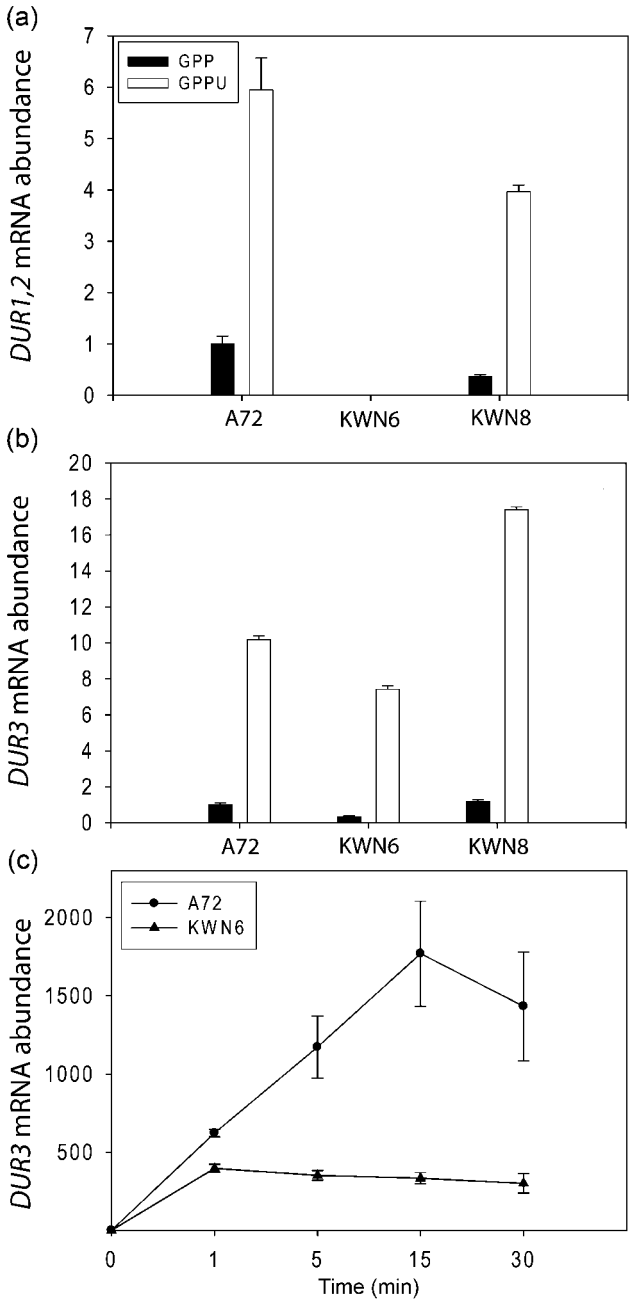

Fig. 2. Urea induction of *DUR1,2* and *DUR3* gene expression. mRNA abundance was determined by qPCR for *DUR1,2* (a) and *DUR3* (b) in *C. albicans* strains A72 (wild-type), KWN6 (*dur1,2*Δ/*dur1,2*Δ) and the reconstituted strain KWN8 (*dur1,2*Δ::*DUR1,2*/*dur1,2*Δ::*DUR1,2*). The cells were grown in defined GPP (filled bars) or GPPU (open bars) medium. (c) Kinetics of induction of *DUR3* in the wild-type strain A72 and the *dur1,2* knockout strain KWN6 in GPU medium.

increased *DUR1,2* mRNA levels 3.8±0.5-fold and *DUR3* mRNA levels 9.2±0.5-fold, compared with GPP. Finally, 10 mM parabanic acid (oxaluric acid), a gratuitous inducer of *DUR1,2* in *S. cerevisiae* (Cooper *et al.*, 1980), also induced *DUR1,2* mRNA levels 6±0.08-fold and *DUR3* mRNA levels 1.8±0.02-fold in *C. albicans* (data not shown).

Transport of [^{14}C]urea into *C. albicans*

The functional importance of Dur3 in urea transport was examined by measuring [^{14}C]urea uptake (Table 3). Preliminary experiments established that optimal uptake was attained at 5 min and gradually decreased thereafter due to catabolism (data not shown). By 5 min, urea uptake levels for a *dur3*Δ mutant were approximately three times lower than those for the wild-type *C. albicans*. Reconstitution of one allele of *DUR3* in the deletion mutant restored urea uptake to wild-type levels (Table 3). This finding is consistent with our observation that the *dur3*Δ mutant grew very slowly on GPU, with urea as the sole nitrogen source. The remaining urea transport in the *dur3*Δ mutant (Table 3) was likely achieved by one or more of the other, non-inducible urea transporters (Fig. 1). Urea transport by Dur3 was energy-dependent in that it was reduced four- to fivefold in the presence of 5 mM sodium azide (Table 3).

Distribution of the urea transporter *DUR3* in fungi

Because the fungal-specific urea transporter *DUR3* resides only 1000 bp upstream of *DUR1,2* on chromosome 1 in *C. albicans*, we expected that its phylogenetic distribution might parallel that of *DUR1,2*, but it did not. *DUR3* orthologues were present as a single copy in all of the 22 ascomycete and basidiomycete genomes searched (Navarathna *et al.*, 2010), except for those of *Ustilago maydis* and *Rhizopus oryzae*, where it was absent, and *Yarrowia lipolytica*, where it was present in two copies. *Y. lipolytica* also has two copies of *DUR1,2*. This gene duplication may have occurred relatively recently, at the species level, resulting in co-duplication of *DUR1,2* and *DUR3*. *DUR3* orthologues were also not detected in three fungi not included in our previous study, *Histoplasma capsulatum*, *Coccidioides immitis* and *Paracoccidioides* sp.

Regulation of *DUR1,2* and *DUR3* expression by other nitrogen sources

We next explored how urea induction of *DUR1,2* and *DUR3* relates to NCR regulation of these genes. For this purpose, *C. albicans* SC5314 cells were grown in defined media containing the well-characterized nitrogen sources proline, glutamine, ammonium sulfate and asparagine, all at 10 mM (Fig. 3). In all cases, expression of *DUR1,2* and *DUR3* was higher in the GP basal media than in YPD medium. Addition of proline or glutamine at 10 mM in the presence of urea significantly repressed expression relative to urea alone (compare GPPU and GPQU with GPU in Fig. 3). However, addition of 10 mM urea further stimulated expression of *DUR1,2* and *DUR3* mRNA (compare GPQU, GPNU and GPNH_4U with GPQ, GPN

Table 3. Urea uptake by wild-type and mutant *C. albicans*

Values shown are radioactivity internalized in $4–5 \times 10^6$ cells after exposure to [^{14}C]urea for 5 min, and are the mean ± SEM of triplicate experiments.

Strain	Treatment	c.p.m.
SC5314 (wild-type)	GP with [^{14}C]urea	2707 ± 660
SC5314 (wild-type)	GP with [^{14}C]urea and NaN_3	517 ± 129
DUR3M4A (*dur3*Δ)	GP with [^{14}C]urea	960 ± 280
DUR3K2A (*dur3*Δ + *DUR3*)	GP with [^{14}C]urea	2698 ± 316

and $GPNH_4$, respectively, in Fig. 3). Notably, the levels of gene expression were highest when urea was the sole nitrogen source added to the basal medium. Therefore, the *DUR1,2* and *DUR3* genes appear to be under limited NCR in that their responsiveness to urea is largely independent of NCR regulation.

Regulation of urea utilization by GATA transcription factors

To further examine the contribution of NCR to regulation of these urea utilization and transporter genes, we compared basal (YPD) and induced (GPU) expression of *DUR1,2* and *DUR3* pairwise in four strains: wild-type *C. albicans* and mutants lacking one or both of the GATA transcription factors Gat1 and Gln3 (Fig. 4). Induction of *DUR1,2* mRNA was not reduced in the *gat1*Δ mutant, was significantly reduced in the *gln3*Δ mutant ($P<0.001$ for *gln3*Δ vs wild-type), and abolished in the double mutant ($P<0.001$ for *gat1*Δ *gln3*Δ vs *gat1*Δ, Fig. 4). Interestingly, urea induction of the transporter *DUR3* was not affected by deletion of *GAT1* or *GLN3* individually, but it was significantly reduced in the double mutant ($P<0.001$ for *gat1*Δ *gln3*Δ vs wild-type). However, urea still induced *DUR3* expression in the double mutant ($P<0.01$ for GPU vs YPD), indicating that other transcriptional regulators are involved.

NCR regulation of *DUR3* involves additional signals

To confirm that, in addition to Gat1 and Gln3, other signalling mechanisms control *DUR1,2* and *DUR3* expression, we tested *DUR1,2* and *DUR3* mRNA levels in the *gat1*Δ, *gln3*Δ and *gat1*Δ *gln3*Δ mutants in the presence of different nitrogen sources. *DUR1,2* mRNA levels were high in *gat1*Δ cells grown in GPU, but addition of glutamine or

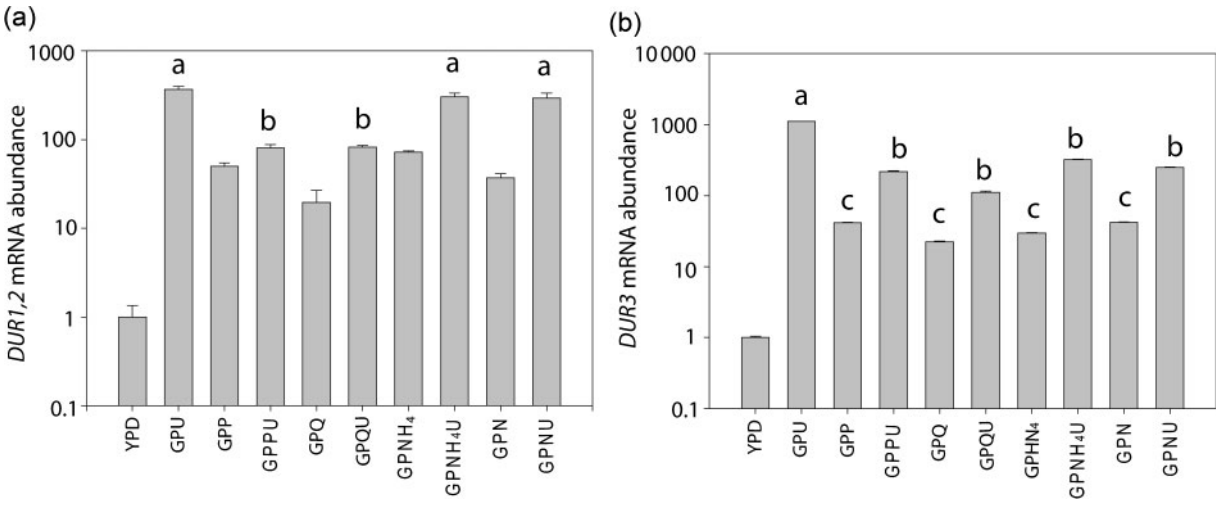

Fig. 3. Effect of nitrogen source on *DUR1,2* and *DUR3* expression in *C. albicans*. DUR1,2 (a) and DUR3 (b) mRNA abundance was determined by qPCR for *C. albicans* SC5314 cells grown with the following additives in defined GP-based media: P, L-proline; U, urea; NH_4, ammonia; Q, L-glutamine; N, asparagine; all at 10 mM. Similar results were obtained when A72 wild-type cells were subjected to the same treatments. Experiments were performed in triplicate; error bars, SEM. Addition of proline and glutamine significantly reduced *DUR1,2* expression (b vs a = $P<0.001$), and addition of L-proline, glutamine, ammonia and asparagine significantly reduced expression of *DUR3* (b vs a = $P<0.001$ by ANOVA) compared with expression levels in GPU. *DUR3* expression was induced by addition of urea, regardless of the nitrogen source tested (c vs b = $P<0.001$, 0.001, 0.05 and 0.001 for GPP, GPQ, GPNH4 and GPN, respectively, by ANOVA).

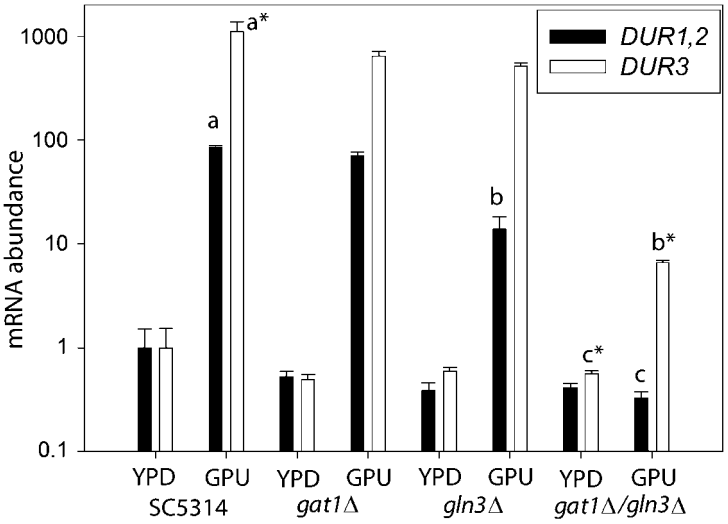

Fig. 4. Role of GATA transcription factors on expression of *DUR1,2* and *DUR3*. The cells were grown in GPU and YPD. Open bars, *DUR3*; filled bars, *DUR1,2*. Lower-case type with an asterisk represents significant changes in *DUR3* expression in GPU relative to expression in the wild-type strain in YPD. Lower-case type without an asterisk indicates significant changes in *DUR1,2* expression in GPU relative to that of the wild-type strain in YPD medium. Experiments were performed in triplicate; error bars, SEM. Compared with the wild-type, the *gln3*Δ and *gln3*Δ *gat1*Δ mutants showed significantly reduced *DUR1,2* expression in GPU ($P<0.001$ for b vs a and c vs a), and *DUR1,2* expression was also significantly lower in the *gln3*Δ *gat1*Δ double mutant than in the *gln3*Δ single mutant ($P<0.001$ for c vs b). In contrast, *DUR3* expression was not affected in the *gln3*Δ and *gat1*Δ single mutants, although it was significantly reduced ($P<0.001$ for b* vs a*) in the *gln3*Δ *gat1*Δ double mutant compared with the wild-type strain grown in GPU. The strains did not differ in expression when grown in YPD.

ammonia significantly reduced its expression ($P<0.001$, Fig. 5a, b). A similar pattern of *DUR1,2* expression was seen in the *gln3*Δ strain grown with GPU and GPU with glutamine ($P<0.002$). However, addition of ammonia did not significantly reduce *DUR1,2* induction. In the *gat1*Δ *gln3*Δ double mutant, urea failed to induce *DUR1,2* expression above basal levels.

As shown in Fig. 4, deletion of *GAT1* or *GLN3* individually did not alter the urea induction of *DUR3* expression. Glutamine and ammonia significantly suppressed *DUR3* induction in the wild-type as well as in the *gat1*Δ and *gln3*Δ single mutants, suggesting that neither GATA transcription factor tested is absolutely required for the induction or repression of this gene (Fig. 5c, d). However, induction of *DUR3* by urea was diminished in the double mutant, and addition of an alternative nitrogen source in this mutant did not further reduce its expression.

DUR3 does not contribute to *C. albicans* virulence in mice

The tail vein injection mouse candidaemia model was used to compare the virulence of the wild-type strain SC5314, the *dur3*Δ mutant DUR3M4A, and the complemented strain DUR3K2A. No significant differences in the survival of mice infected with the three strains were observed (data not shown).

DISCUSSION

Although urea metabolism in the hemiascomycetes diverges from that of other fungi in using the biotin-containing enzyme Dur1,2 rather than the nickel-containing urease (Navarathna *et al.*, 2010), these fungi have in common a number of putative urea transporters. Among these, we have identified Dur3 as the major urea-inducible transporter in *C. albicans*. Dur3 is responsible for ~80 % of the energy-dependent urea transport into *C. albicans* in basal medium, and presumably accounts for a much higher percentage when induced. The expression of mRNAs for several non-inducible urea transporters in *C. albicans* (Fig. 1) is consistent with our observation that the *dur3*Δ mutant exhibited undiminished pathogenicity in a mouse model of disseminated candidiasis. It is also likely that sufficient amounts of other nitrogen sources are available for growth, so urea uptake is not limiting. The fact that the *dur3*Δ mutant exhibited undiminished pathogenicity is totally consistent with our previous work showing that Dur1,2 is needed for *C. albicans* to escape from macrophages. In that case, the urea was produced intracellularly from L-arginine. Thus, no import of exogenous urea was needed.

Notably, all other urea transporters are expressed at lower mRNA levels than *DUR3*. Indeed, some of these putative urea transporters may not be functional or may have other dominant substrates. For instance, Dur3 in *S. cerevisiae* has recently been shown to be a functional boron transporter (Nozawa *et al.*, 2006).

Although urea can serve as a nitrogen source for *C. albicans* (Dastidar *et al.*, 1967), the inducible transporter Dur3 and the catabolic enzyme Dur1,2 do not exhibit classical NCR regulation. When rich nitrogen sources such as ammonia and asparagine are available, cells preferentially utilize these sources, while repressing genes that are responsible for

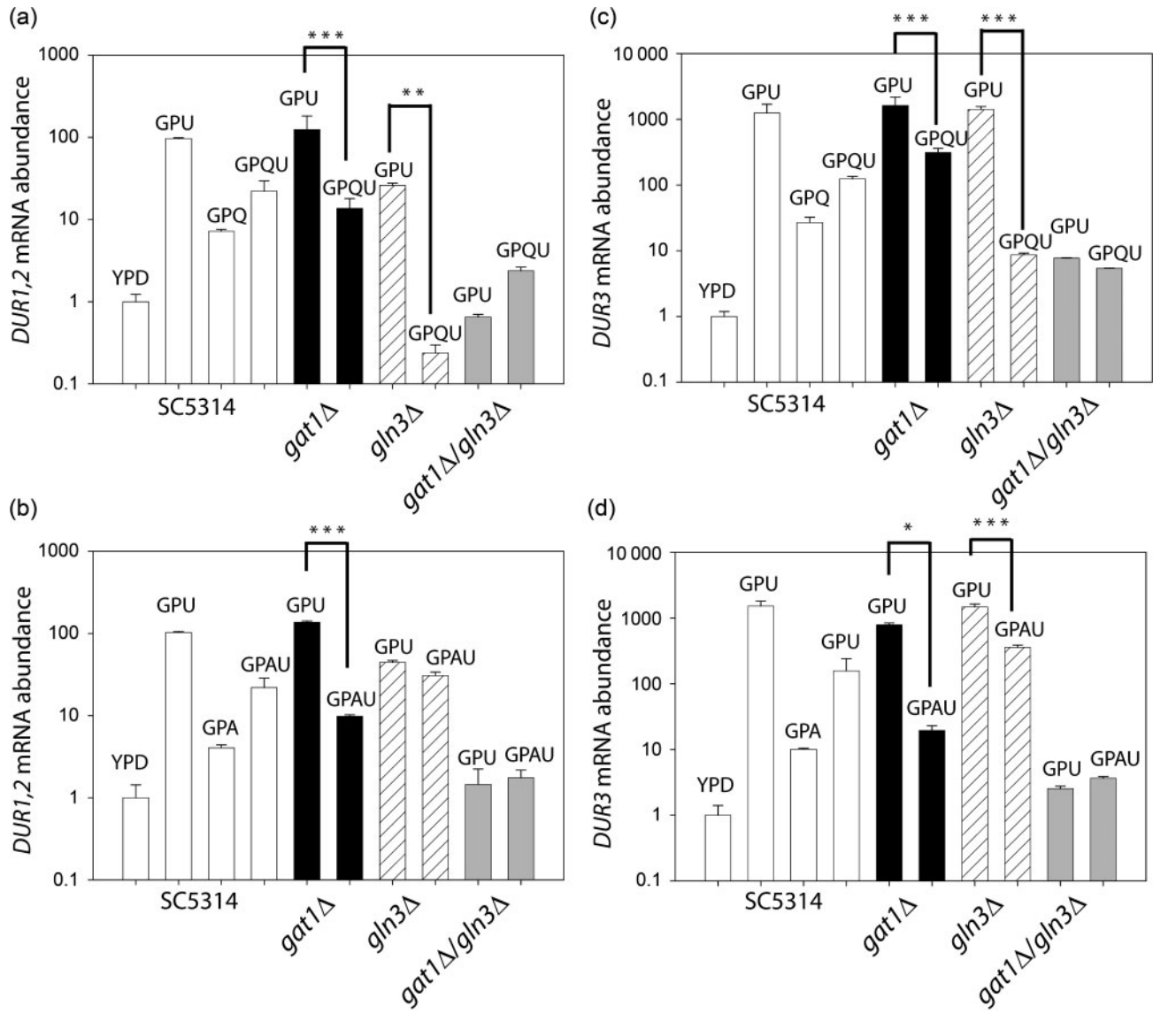

Fig. 5. Role of GATA transcription factors in the gene expression of *DUR1,2* (a, b) and *DUR3* (c, d) with or without other nitrogen sources (10 mM) in the growth media containing urea (10 mM). (a, c) Gene expression was quantified in the presence of glutamine (Q). (b, d) Gene expression was quantified in the presence of ammonia. *DUR1,2* expression (a, b) and *DUR3* expression (c, d) in the indicated transcription factor mutants was compared with that in the wild-type strain SC5314. Experiments were performed in triplicate; error bars, SEM. In the *gat1*Δ mutant, addition of glutamine and ammonia caused a significant reduction of *DUR1,2* expression ($P<0.001$) compared with the levels in GPU medium. Both the *gat1*Δ and the *gln3*Δ mutant exhibited reduced *DUR3* expression when glutamine ($P<0.001$) or ammonia ($P<0.05$ for *gat1*Δ and 0.001 for *gln3*Δ) was added to GPU. *, $P<0.05$; **, $P<0.002$; ***, $P<0.001$.

breaking down poorer nitrogen sources such as proline, arginine and urea (Cooper, 1982). When cells are starved for nitrogen, these NCR-regulated genes are induced. In *S. cerevisiae*, urea amidolyase is subject to NCR (Cox *et al.*, 2000b), and we have previously shown in *C. albicans* that 5–10 mM ammonium salts prevent germ tube formation induced by GlcNAc, arginine or urea, but not that induced by 5 % CO_2 (Ghosh *et al.*, 2009). These results were expected, because *C. albicans DUR1,2* has at least five (A/T) GATA(A/G) sites upstream of the ORF (Ghosh *et al.*, 2009), and these are the putative binding sites for the GATA transcription factors Gln3 and Gat1, which may mediate gene expression under inducing conditions (Dabas & Morschhäuser, 2007). These sites could mediate both direct and indirect NCR (via ammonium ions) of *DUR1,2* expression. However, we show here that urea induction of these genes in GP medium is maintained in the presence of rich and poor nitrogen sources, including proline,

glutamine, NH_4 and asparagine. The putative GATA sites upstream of *DUR3* probably contribute to its induction by urea, although residual induction by urea in the *gat1*Δ *gln3*Δ mutant suggests that additional transcription factors mediate *DUR3* induction by urea.

In *S. cerevisiae*, the direct inducer for *DUR1,2* is allophanate, also known as urea carboxylate, rather than urea itself (Cooper, 1982). Allophanate is the chemical intermediate for the multifunctional Dur1,2. In *C. albicans*, allophanate may also be an inducer for *DUR1,2*, although urea induction of *DUR3* must involve a different mechanism. This conclusion is based on the observation that the *dur1,2*Δ mutant showed the same relative initial *DUR3* induction by urea as the wild-type (Fig. 2b). However, following *DUR3* expression over time after the introduction of urea revealed that the *dur1,2*Δ mutant lacked the slow secondary induction of *DUR3* expression seen for wild-type cells grown in GPPU (Fig. 2c). Presumably, the longer growth time in GPPU permitted accumulation of another, less efficient inducer (potentially allophanate) and/or the lifting of a partial NCR caused by the metabolism of L-proline.

The expression of *DUR1,2* and *DUR3* was minimal in the complex medium YPD, suggesting that these genes could be subject to classical NCR. Although the high constitutive expression of both genes in basal medium containing urea was significantly repressed by addition of either good (ammonia) or poor (proline) nitrogen sources (Limjindaporn *et al.*, 2003; Marzluf, 1997), disruption of the NCR transcription factor Gat1 did not prevent induction of *DUR1,2* by urea or its repression by either nitrogen source. Similarly, *DUR3* induction by urea was refractory to deletion of Gat1 or Gln3, and addition of nitrogen sources significantly repressed *DUR3* expression in the presence of urea (Fig. 5c, d). Yet, induction of both genes by urea was dramatically decreased in the *gat1*Δ *gln3*Δ double mutant, and this strain was not subject to further repression by ammonia or Gln. These observations indicate that the GATA transcription factors Gat1 and Gln3 play positive roles in the urea induction of these genes, and that another NCR mechanism controls the negative regulation of *DUR1,2* and *DUR3* expression.

Complexity in the adaptive responses to varying environmental nitrogen sources is not unique to *C. albicans*. *Aspergillus* AreA and *Neurospora* NIT2 play primary roles in derepressing NCR (Caddick *et al.*, 1994). *Aspergillus amdS* encodes an acetamidase that releases ammonia. This gene is controlled by pathway-specific gene regulation as well as general regulatory proteins (Davis *et al.*, 1993). Gln3 of *S. cerevisiae* resembles AreA and NIT2 as a positive-acting regulator of transcription (Cooper *et al.*, 1990). *Aspergillus nirA* encodes a nitrate reductase that requires AreA for induction. A mutation of *nirA* has been shown to overcome the requirement for AreA, suggesting that both specific and general regulation pathways are required for nitrogen metabolism in *Aspergillus* (Cove, 1979). These insights from other fungi will guide our future studies of the signalling mechanisms that regulate urea transport in *C. albicans*.

Although deletion of the inducible urea transporter Dur3 did not decrease virulence in a standard mouse candidaemia model, inducible acquisition of urea may be important for other aspects of *C. albicans* pathogenesis. Nitrogen metabolism contributes to the pathogenicity of *Aspergillus fumigatus* (Krappmann & Braus, 2005). Urease is a virulence factor in *Cryptococcus neoformans* (Cox *et al.*, 2000a) and *Coccidiodes immitis* (Yu *et al.*, 1997). In addition, urease is a well-established virulence factor in *H. pylori*. A urease mutant is unable to survive in co-culture with macrophages, whereas the urease in wild-type *H. pylori* prevents phagosome maturation (Schwartz & Allen, 2006). We know that urea metabolism provides similar protection from clearance by macrophages in *C. albicans* (Ghosh *et al.*, 2009). Future studies will further examine the role of urea transport and metabolism in pathogenesis and examine the virulence of *dur1,2*Δ mutants. We will also explore the potential for urea catabolism to contribute to colonization of the kidney, where the organism could be exposed to higher concentrations of urea than in the bloodstream.

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REFERENCES

Caddick, M. X., Peters, D. & Platt, A. (1994). Nitrogen regulation in fungi. *Antonie van Leeuwenhoek* **65**, 169–177.

Cole, G. T. (1997). Ammonia production by *Coccidiodes immitis* and its possible significance to the host–fungus interplay. In *Host–Fungus Interplay*, pp. 247–263. Edited by D. O. Stevens. Bethesda, MD: National Foundation for Infectious Diseases.

Collart, M. A. & Struhl, K. (1994). NOT1(CDC39), NOT2(CDC36), NOT3, and NOT4 encode a global-negative regulator of transcription that differentially affects TATA-element utilization. *Genes Dev* **8**, 525–537.

Cooper, T. G. (1982). Nitrogen metabolism in *Saccharomyces cerevisiae*. In *The Molecular Biology of the Yeast Saccharomyces*, pp. 39–99. Edited by J. N. Strathern, E. W. Jones & J. R. Broach. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory.

Cooper, T. G., Lam, C. & Turoscy, V. (1980). Structural analysis of the *dur* loci in *S. cerevisiae*: two domains of a single multifunctional gene. *Genetics* **94**, 555–580.

Cooper, T. G., Ferguson, D., Rai, R. & Bysani, N. (1990). The *GLN3* gene product is required for transcriptional activation of allantoin system gene expression in *Saccharomyces cerevisiae*. *J Bacteriol* **172**, 1014–1018.

Cove, D. J. (1979). Genetic studies of nitrate assimilation in *Aspergillus nidulans*. *Biol Rev Camb Philos Soc* **54**, 291–327.

Cox, G. M., Mukherjee, J., Cole, G. T., Casadevall, A. & Perfect, J. R. (2000a). Urease as a virulence factor in experimental cryptococcosis. *Infect Immun* **68**, 443–448.

Cox, K. H., Rai, R., Distler, M., Daugherty, J. R., Coffman, J. A. & Cooper, T. G. (2000b). *Saccharomyces cerevisiae* GATA sequences function as TATA elements during nitrogen catabolite repression and when Gln3p is excluded from the nucleus by overproduction of Ure2p. *J Biol Chem* **275**, 17611–17618.

Dabas, N. & Morschhäuser, J. (2007). Control of ammonium permease expression and filamentous growth by the GATA transcription factors *GLN3* and *GAT1* in *Candida albicans*. *Eukaryot Cell* **6**, 875–888.

Dastidar, S. G., Purandare, N. M. & Desai, S. C. (1967). Growth requriements of *Candida* species. *Indian J Exp Biol* **5**, 228–232.

Davis, M. A., Kelly, J. M. & Hynes, M. J. (1993). Fungal catabolic gene regulation: molecular genetic analysis of the *amdS* gene of *Aspergillus nidulans*. *Genetica* **90**, 133–145.

Di Carlo, F. J., Schultz, A. S. & Kent, A. M. (1953). The mechanism of allantoin catabolism by yeast. *Arch Biochem Biophys* **44**, 468–474.

Dunkel, N., Blass, J., Rogers, P. D. & Morschhäuser, J. (2008). Mutations in the multi-drug resistance regulator *MRR1*, followed by loss of heterozygosity, are the main cause of *MDR1* overexpression in fluconazole-resistant *Candida albicans* strains. *Mol Microbiol* **69**, 827–840.

Eaton, K. A., Brooks, C. L., Morgan, D. R. & Krakowka, S. (1991). Essential role of urease in pathogenesis of gastritis induced by *Helicobacter pylori* in gnotobiotic piglets. *Infect Immun* **59**, 2470–2475.

Gillum, A. M., Tsay, E. Y. & Kirsch, D. R. (1984). Isolation of the *Candida albicans* gene for orotidine-5′-phosphate decarboxylase by complementation of *S. cerevisiae ura3* and *E. coli pyrF* mutations. *Mol Gen Genet* **198**, 179–182.

Ghosh, S., Navarathna, D. H., Roberts, D. D., Cooper, J. T., Atkin, A. L., Petro, T. M. & Nickerson, K. W. (2009). Arginine-induced germ tube formation in *Candida albicans* is essential for escape from murine macrophage line RAW 264.7. *Infect Immun* **77**, 1596–1605.

Jones, B. D., Lockatell, C. V., Johnson, D. E., Warren, J. W. & Mobley, H. L. (1990). Construction of a urease-negative mutant of *Proteus mirabilis*: analysis of virulence in a mouse model of ascending urinary tract infection. *Infect Immun* **58**, 1120–1123.

Köhrer, K. & Domdey, H. (1991). Preparation of high molecular weight RNA. *Methods Enzymol* **194**, 398–405.

Krappmann, S. & Braus, G. H. (2005). Nitrogen metabolism of *Aspergillus* and its role in pathogenicity. *Med Mycol* **43** (Suppl. 1), S31–S40.

Kulkarni, R. K. & Nickerson, K. W. (1981). Nutritional control of dimorphism in *Ceratocystis ulmi*. *Exp Mycol* **5**, 148–154.

Limjindaporn, T., Khalaf, R. A. & Fonzi, W. A. (2003). Nitrogen metabolism and virulence of *Candida albicans* require the GATA-type transcriptional activator encoded by *GAT1*. *Mol Microbiol* **50**, 993–1004.

Marzluf, G. A. (1997). Genetic regulation of nitrogen metabolism in the fungi. *Microbiol Mol Biol Rev* **61**, 17–32.

Navarathna, D. H. & Roberts, D. D. (2010). *Candida albicans* heme oxygenase and its product CO contribute to pathogenesis of candidemia and alter systemic chemokine and cytokine expression. *Free Radic Biol Med* **49**, 1561–1573.

Navarathna, D. H., Hornby, J. M., Hoerrmann, N., Parkhurst, A. M., Duhamel, G. E. & Nickerson, K. W. (2005). Enhanced pathogenicity of *Candida albicans* pre-treated with subinhibitory concentrations of fluconazole in a mouse model of disseminated candidiasis. *J Antimicrob Chemother* **56**, 1156–1159.

Navarathna, D. H., Nickerson, K. W., Duhamel, G. E., Jerrels, T. R. & Petro, T. M. (2007). Exogenous farnesol interferes with the normal progression of cytokine expression during candidiasis in a mouse model. *Infect Immun* **75**, 4006–4011.

Navarathna, D. H., Harris, S. D., Roberts, D. D. & Nickerson, K. W. (2010). Evolutionary aspects of urea utilization by fungi. *FEMS Yeast Res* **10**, 209–213.

Nozawa, A., Takano, J., Kobayashi, M., von Wiren, N. & Fujiwara, T. (2006). Roles of BOR1, DUR3, and FPS1 in boron transport and tolerance in *Saccharomyces cerevisiae*. *FEMS Microbiol Lett* **262**, 216–222.

Odds, F. C. (1988). *Candida and Candidiasis*, 2nd edn. London: Bailliere Tindall.

Pendrak, M. L., Yan, S. S. & Roberts, D. D. (2004a). Sensing the host environment: recognition of hemoglobin by the pathogenic yeast *Candida albicans*. *Arch Biochem Biophys* **426**, 148–156.

Pendrak, M. L., Yan, S. S. & Roberts, D. D. (2004b). Hemoglobin regulates expression of an activator of mating-type locus α genes in *Candida albicans*. *Eukaryot Cell* **3**, 764–775.

Pendrak, M. L., Chao, M. P., Yan, S. S. & Roberts, D. D. (2004c). Heme oxygenase in *Candida albicans* is regulated by hemoglobin and is necessary for metabolism of exogenous heme and hemoglobin to α-biliverdin. *J Biol Chem* **279**, 3426–3433.

Pfaffl, M. W. (2001). A new mathematical model for relative quantification in real-time RT-PCR. *Nucleic Acids Res* **29**, e45.

Reuß, O., Vik, Å., Kolter, R. & Morschhäuser, J. (2004). The *SAT1* flipper, an optimized tool for gene disruption in *Candida albicans*. *Gene* **341**, 119–127.

Roon, R. J. & Levenberg, B. (1972). Urea amidolyase. I. Properties of the enzyme from *Candida utilis*. *J Biol Chem* **247**, 4107–4113.

Roon, R. J., Hampshire, J. & Levenberg, B. (1972). Urea amidolyase. The involvement of biotin in urea cleavage. *J Biol Chem* **247**, 7539–7545.

Schwartz, J. T. & Allen, L. A. (2006). Role of urease in megasome formation and *Helicobacter pylori* survival in macrophages. *J Leukoc Biol* **79**, 1214–1225.

Sentheshanmuganathan, S. & Nickerson, W. J. (1962). Nutritional control of cellular form in *Trigonopsis variabilis*. *J Gen Microbiol* **27**, 437–449.

Yu, J. J., Smithson, S. L., Thomas, P. W., Kirkland, T. N. & Cole, G. T. (1997). Isolation and characterization of the urease gene (*URE*) from the pathogenic fungus *Coccidioides immitis*. *Gene* **198**, 387–391.

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