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NRG1 represses yeast–hypha morphogenesis and hypha-specific gene expression in *Candida albicans*

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We have characterized CaNrg1 from *Candida albicans*, the major fungal pathogen in humans. CaNrg1 contains a zinc finger domain that is conserved in transcriptional regulators from fungi to humans. It is most closely related to ScNrg1, which represses transcription in a Tup1-dependent fashion in *Saccharomyces cerevisiae*. Inactivation of CaNrg1 in *C.albicans* causes filamentous and invasive growth, derepresses hypha-specific genes, increases sensitivity to some stresses and attenuates virulence. A *tup1* mutant displays similar phenotypes. However, unlike *tup1* cells, *nrg1* cells can form normal hyphae, generate chlamydospores at normal rates and grow at 42°C. Transcript profiling of 2002 *C.albicans* genes reveals that CaNrg1 represses a subset of CaTup1-regulated genes, which includes known hypha-specific genes and other virulence factors. Most of these genes contain an Nrg1 response element (NRE) in their promoter. CaNrg1 interacts specifically with an NRE *in vitro*. Also, deletion of two NREs from the *ALS8* promoter releases it from Nrg1-mediated repression. Hence, CaNrg1 is a transcriptional repressor that appears to target CaTup1 to a distinct set of virulence-related functions, including yeast–hypha morphogenesis.

Keywords: *Candida albicans*/NRG1 gene/transcript profiling/transcriptional repression/yeast–hypha morphogenesis

Introduction

Morphological transitions between unicellular and filamentous growth forms contribute significantly to the virulence of fungal pathogens. The major plant pathogens *Ustilago maydis* and *Magnaporthe grisae* form hyphae that penetrate their hosts in the early stages of infection (Hartmann *et al.*, 1996; Hamer and Talbot, 1998). Transitions from avirulent filamentous forms to virulent unicellular forms are essential for the establishment of *Histoplasma capsulatum* and *Paracoccidioides brasiliensis* infections in humans (da Silva *et al.*, 1999; Maresca and Kobayashi, 2000). The most prevalent fungal pathogen of humans, *Candida albicans*, undergoes reversible morphological transitions between unicellular yeast-like, pseudohyphal and hyphal growth forms, which contribute to disease establishment and progression (Odds, 1994; Brown and Gow, 1999). The yeast form is probably disseminated more effectively than filamentous forms, whereas the hyphal form seems better adapted to penetrate tissue and is thought to help the fungus evade host defences (Sherwood *et al.*, 1992; Lo *et al.*, 1997).

Numerous parameters influence the yeast–hypha transition in *C.albicans* (Odds, 1988), and these presumably reflect the variety of signals detected by the fungus in the different microenvironments it encounters within its human host (Brown and Gow, 1999). Hyphal development is subject to both positive and negative regulation. Positive regulation is mediated by multiple signalling pathways including mitogen-activated protein kinase (MAPK), cAMP and pH signalling pathways (Brown and Gow, 1999; Ernst, 2000). Negative regulation is dependent upon the factor CaTup1 (Braun and Johnson, 1997). Inactivation of CaTup1 leads to constitutive filamentous growth and the derepression of hypha-specific genes (Braun and Johnson, 1997, 2000; Braun *et al.*, 2000; Brown *et al.*, 2000). CaTup1 appears to act independently of the MAPK and cAMP pathways to regulate morphogenesis (Braun and Johnson, 2000), but the mechanisms by which CaTup1 operates remain obscure. In *Saccharomyces cerevisiae*, ScTup1 represses the transcription of sets of functionally related genes, interacting with their promoters indirectly through specific DNA-binding proteins (Keleher *et al.*, 1992; Treitel and Carlson, 1995; Park *et al.*, 1999; Smith and Johnson, 2000). CaTup1 might operate in a similar way to repress the expression of hypha-specific genes and control morphogenesis in *C.albicans*.

In this study, we report the identification of CaNrg1, which represses hyphal development and hypha-specific gene expression in *C.albicans*. Our data suggest that CaNrg1 achieves this by targeting CaTup1 to hypha-specific genes.

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		% Identity
CaNrg1	RKHV-----CKV--CSRSFTTSGHLARHNR-IHTGERKHQCPWPTCEARFARQDNCNQHYKTHTNGKNK	
ScNrg1	RKYI-----CKI--CARGFTTSGHLARHNR-IHTGEKNHCCPYKGCTQRFSRHDNCLQHYRTHLKKGQ	67%
ScNrg2	TRHF-----CKI--CSTGFTTSGHLSRHNR-IHTGEKNHICPHEGCGQRFSRHDNCNQHYRTHAN-KKK	62%
ScMsn2	KPFH-----CHI--CPKSFKRSEHLKRHVRSVHSNERPFACH--ICDKKFSRSDNLSQHIKTH---KKH	46%
ScMig1	RPHA-----CPI--CHRAFHRLEHQTRHMR-IHTGEKPHACDFPGCVKRFSRSDELTRHRRIHTNSHPR	44%
HuEGRTF	RSHI-----CSHPGCGKTYFKSSHLKAHTR-THTGEKPFSCSWKGCERRFARSDELSRHRRTHTGEKKF	44%
HuCKLP	RIHY-----CDYPGCTKVYTKSSHLKAHLR-THTGEKPYKCTWEGCDWRFARSDELTRHYRKHTGAK	43%
KlMIG1	RPYV-----CPI--CQRGFHRLEHQTRHIR-THTGERPHACDFPGCSKRFSRSDELTRHRRIHDSDKPK	41%
HuWTP	RHTGVKPFQCKT--CQRKFSRSDHLKTHTR-THTGEKPFSCRWPSCQKKFARSDELVRHHNMHQRNMTK	39%
AnCREA	RPYK-----CPL--CERAFHRLEHQTRHIR-THTGEKPHACQFPGCSKRFSRSDELTRHSRIHNNPNSR	38%
	--C----C--------H---H------------C----C------------H---H--	

Fig. 1. *Candida albicans* Nrg1 is related to C_2H_2 zinc finger proteins from other species. Alignment of the CaNrg1 C_2H_2 domain (residues 226–286) with related fungal and mammalian proteins: ScNrg1, *S.cerevisiae* Nrg1; ScNrg2, *S.cerevisiae* Nrg2; ScMsn2, *S.cerevisiae* Msn2p; ScMig1, *S.cerevisiae* Mig1; HuEGRTF, human early growth response transcription factor; HuCKLP, human colon Kruppel-like protein; KlMIG1, *Kluyveromyces lactis* MIG1 protein; HuWTP, human Wilms' tumour protein; AnCREA, *Aspergillus niger* CREA protein. Sequence identities to CaNrg1 are provided (%).

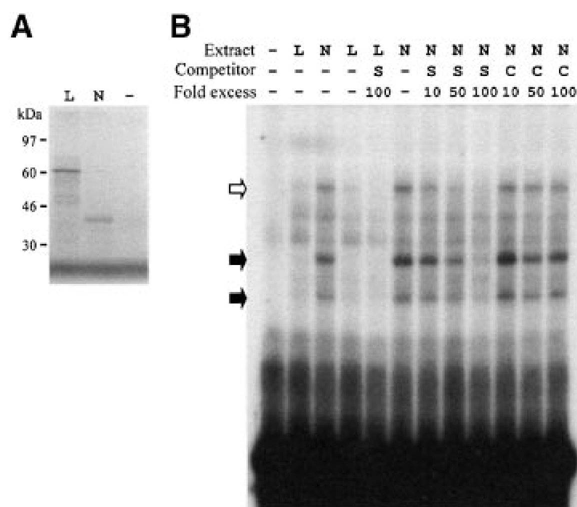

Fig. 2. Nrg1 is a sequence-specific DNA-binding protein. (**A**) Synthesis of *in vitro* transcribed–translated CaNrg1. SDS–polyacrylamide gel of ^{35}S-labelled products with a rabbit *in vitro* transcription–translation mix: control reaction with luciferase plasmid, L; reaction with *NRG1* plasmid, N; reaction with no plasmid, –. (**B**) Gel shift assays of complexes formed with a ^{32}P-labelled double-stranded C_4T-containing oligonucleotide: Nrg1 extract, N; control *in vitro* transcription–translation luciferase extract, L; competition with the same unlabelled C_4T oligonucleotide, S; competition with a control oligonucleotide lacking C_4T, C. Numbers represent fold excess of competitor. A putative rabbit STRE-specific complex is highlighted by the open arrow, and Nrg1-dependent, sequence-specific complexes are highlighted by the closed arrows.

Results

Identification of C.albicans Nrg1

The *HYR1* and *ALS8* genes are activated specifically during hyphal development in *C.albicans* (Bailey *et al.*, 1996; Leng *et al.*, 2001). Both promoters contain the sequence C_4T (Leng, 1999), which corresponds to the stress response element (STRE) in *S.cerevisiae* (Marchler *et al.*, 1993). Since many conditions that promote yeast–hypha morphogenesis impose a stress upon the *C.albicans* cell (Odds, 1988; Brown and Gow, 1999), we reasoned that C_4T elements might contribute to the regulation of hypha-specific genes. Hence, we sought *C.albicans* factors that interact specifically with C_4T.

A *C.albicans* cDNA library was screened for proteins that interact specifically with a ^{32}P-labelled C_4T-containing oligonucleotide. The screen yielded a cDNA that reacted reproducibly with the C_4T-containing oligonucleotide, but not with three different control oligonucleotides lacking C_4T. This cDNA was used to isolate the corresponding gene.

The 310 amino acid sequence encoded by this gene is most similar to *S.cerevisiae* Nrg1 (Figure 1), and hence it was named CaNrg1. The overall level of sequence identity between CaNrg1 and ScNrg1 is low (22%), but this rises to 67% within a $(C_2H_2)_2$ zinc finger region from residues 230 to 280. This region is closely related to the zinc fingers of several fungal transcription factors that are important regulators of carbon assimilation and stress responses (Nehlin and Ronne, 1990; Lundin *et al.*, 1994; Martinez-Pastor *et al.*, 1996; Park *et al.*, 1999). CaNrg1 is also related to early growth response transcription factor, colon Kruppel-like protein and Wilms' tumour protein, which regulate important developmental processes in humans (Gessler *et al.*, 1990; Blok *et al.*, 1995; Shi *et al.*, 1999).

Gel shift assays were performed to confirm the DNA binding specificity of CaNrg1. Synthetic CaNrg1, made by *in vitro* transcription–translation, was close to its predicted mass of 34 kDa (Figure 2A). CaNrg1 extracts formed two complexes with the C_4T-containing oligonucleotide that were not formed by control extracts lacking CaNrg1 (Figure 2B). These complexes were competed out by unlabelled C_4T-containing oligonucleotide, but not by an oligonucleotide lacking C_4T (Figure 2B). Hence, CaNrg1 can interact specifically with a C_4T-containing sequence *in vitro*, like ScNrg1 (Park *et al.*, 1999).

Morphology of C.albicans nrg1 mutants

Both *CaNRG1* alleles in this diploid fungus were inactivated by mutating the *CaNRG1* start codon (ATG→GTG) and deleting 438 bp of the coding region. Independent *nrg1* mutations were introduced into CAI4 (*ura3/ura3*) and CAI8 (*ura3/ura3*, *ade2/ade2*) backgrounds (Table I). *NRG1* mRNA was undetectable in homozygous *nrg1* mutants (not shown).

Table I. *Candida albicans* strains

Strain	Genotype	Scource
SC5314	wild type	Gillum *et al*. (1984)
CAF2-1	*URA3/ura3::λ imm434*	Fonzi and Irwin (1993)
CAI4	*ura3::λ imm434/ura3::λ imm434*	Fonzi and Irwin (1993)
CAI8	*ura3::λ imm434/ura3::λ imm434, ade2::hisG/ade2::hisG*	Fonzi and Irwin (1993)
BCA2-10	*ura3::λ imm434/ura3::λ imm434, tup1::hisG/tup1::hisG-URA3-hisG*	Braun and Johnson (1997)
LOZ124	*ura3::λ imm434/ura3::λ imm434, his1::hisG/his1::hisG, mig1::HIS1/mig1::hisG-URA3-hisG*	Zaragoza *et al*. (2000)
MMC1	*ura3::λ imm434/ura3::λ imm434, NRG1/nrg1::hisG-URA3-hisG*	this study
MMC2	*ura3::λ imm434/ura3::λ imm434, NRG1/nrg1::hisG*	this study
MMC3	*ura3::λ imm434/ura3::λ imm434, nrg1::hisG-URA3-hisG/nrg1::hisG*	this study
MMC4	*ura3::λ imm434/ura3::λ imm434, nrg1::hisG/nrg1::hisG*	this study
MMC5	*ura3::λ imm434/ura3::λ imm434, nrg1::hisG/nrg1::hisG, RP10-MET3-NRG1*	this study
MMC6	*ura3::λ imm434/ura3::λ imm434, ade2::hisG/ade2::hisG, NRG1/nrg1::hisG-URA3-hisG*	this study
MMC7	*ura3::λ imm434/ura3::λ imm434, ade2::hisG/ade2::hisG, NRG1/nrg1::hisG*	this study
MMC8	*ura3::λ imm434/ura3::λ imm434, ade2::hisG/ade2::hisG, nrg1::hisG-URA3-hisG/nrg1::hisG*	this study
MMC9	*ura3::λ imm434/ura3::λ imm434, ade2::hisG/ade2::hisG, nrg1::hisG/nrg1::hisG*	this study

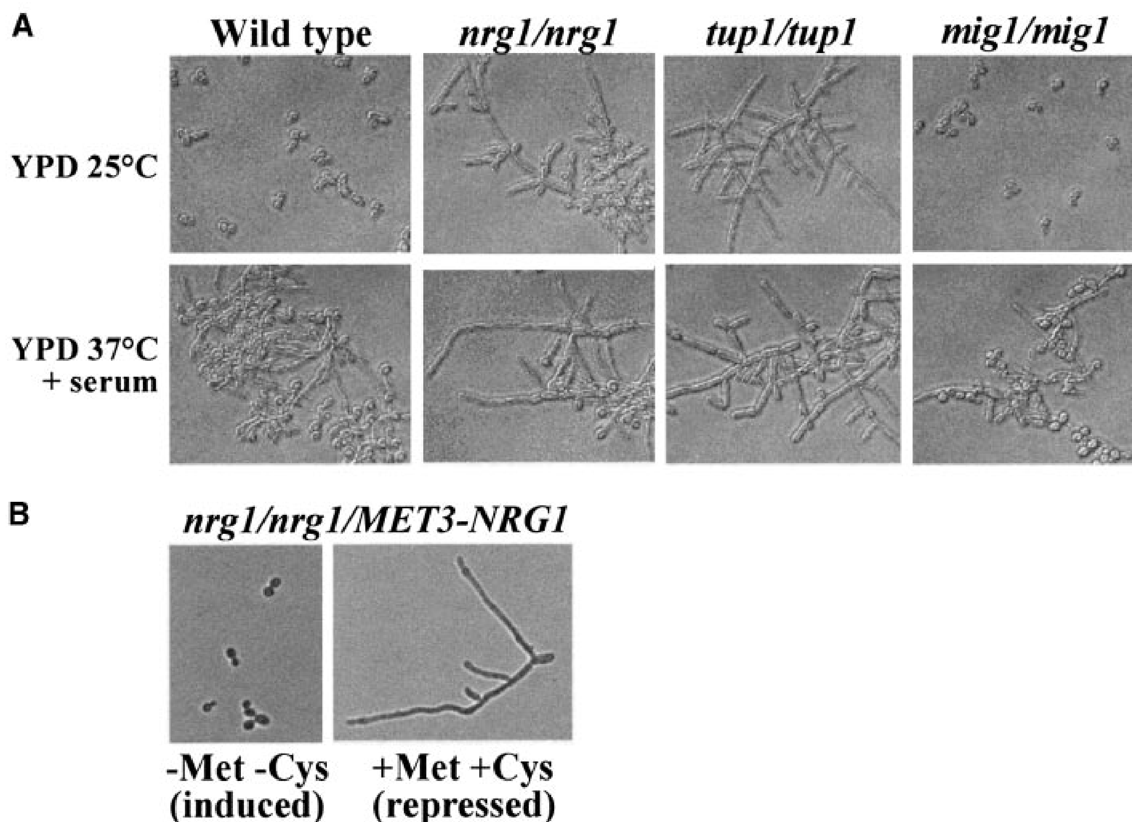

Fig. 3. Inactivation or depletion of *NRG1* derepresses filamentous growth in *C.albicans*. (**A**) Cells were grown in YPD at 25°C (upper panels) or YPD containing 20% serum at 37°C (lower panels): wild-type, CAF2-1; *nrg1*, MMC3; *tup1*, BCa2-10; *mig1*, LOZ124. (**B**) MMC5 cells (*nrg1/nrg1, MET3-NRG1*) growing in SC lacking or containing 10 mM methionine (Met) and cysteine (Cys).

Homozygous *C.albicans nrg1* mutants grew in filamentous forms under conditions that normally promote the growth of yeast cells (Figure 3A). The mutants formed chains of relatively short pseudohyphal cells in YPD at 25°C, and more extended pseudohyphae in synthetic complete medium. They also formed characteristically wrinkly colonies on YPD agar.

To confirm that these phenotypes correlated with the loss of CaNrg1, the *CaNRG1* gene was placed under the control of the *CaMET3* promoter, which is repressed by methionine and cysteine (Care *et al*., 1999). The *nrg1/nrg1/MET3-NRG1* strain (Table I) grew normally in the yeast form in the absence of methionine and cysteine, but formed pseudohyphae following methionine and cysteine addition (Figure 3B). Therefore, CaNrg1 promotes growth of *C. albicans* in the yeast form.

Phenotypes of C.albicans nrg1, mig1 and tup1 mutants

By analogy with their homologues in *S.cerevisiae*, CaNrg1 and CaMig1 are probably CaTup1 targeting proteins. Therefore, we compared the phenotypes of homozygous

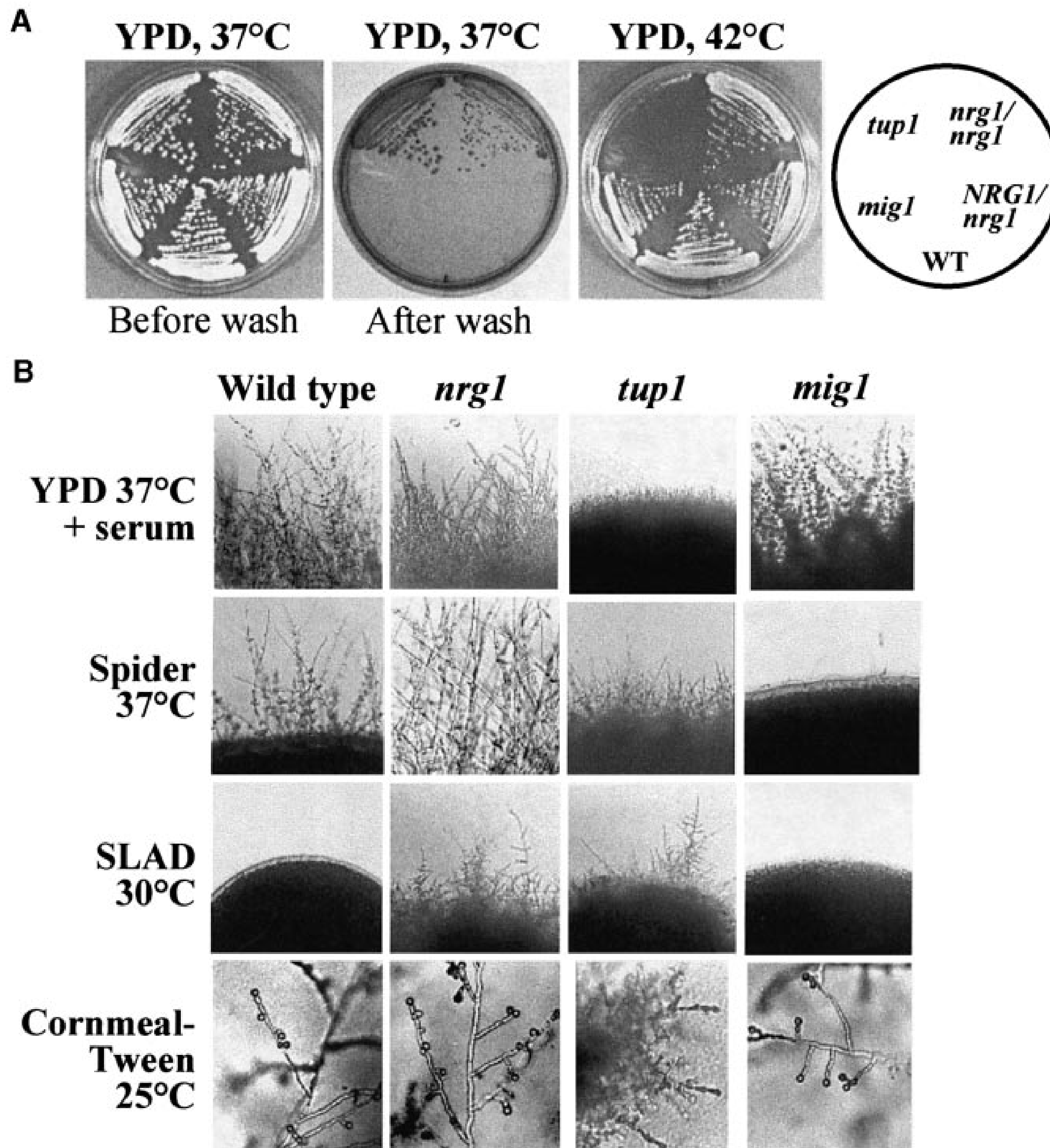

Fig. 4. *Candida albicans nrg1* and *tup1* mutants display subtly different phenotypes. (**A**) Agar invasion (revealed after washing cells from the surface of agar plates) and temperature effects upon growth: wild-type, CAF2-1; *NRG1/nrg1*, MMC1; *nrg1/nrg1*, MMC3; *tup1/tup1*, BCa2-10; *mig1/mig1*, LOZ124. (**B**) Colony fringes of mutants grown on different solid media: 3 days at 37°C on YPD agar containing 20% serum; 6 days at 37°C on Spider agar; 4 days at 30°C on SLAD agar; chlamydospore formation after 7 days at 25°C on cornmeal–Tween agar under coverslips. Homozygous strains are as described in (A).

C.albicans nrg1, *tup1* and *mig1* mutants. Wild-type and *mig1* cells grew normally in the yeast form in YPD at 25°C, and formed normal germ tubes (the progenitors of hyphae) following serum stimulation (Figure 3A). In contrast, *nrg1* and *tup1* cells formed pseudohyphae in YPD at 25°C. This was consistent with previously reported phenotypes for *tup1* and *mig1* cells (Braun and Johnson, 1997; Zaragoza *et al*., 2000). The *C.albicans tup1* and *nrg1* mutants invaded YPD agar constitutively, unlike wild-type and *mig1* cells (Figure 4A). In addition, *tup1* and *nrg1* colonies formed filaments at an enhanced rate on low ammonia SLAD medium compared with wild-type and *mig1* colonies (Figure 4B). Hence, *nrg1* and *tup1* mutants displayed related morphological and invasive phenotypes, suggesting that they might play similar roles in these processes.

Further analyses revealed subtle morphological differences between the *nrg1* and *tup1* mutants. The *tup1* mutant remained fixed in a pseudohyphal morphology, whereas *nrg1* cells formed true hyphae following serum stimulation (Figure 3A) or pH induction (not shown). Similarly, wild-type and *nrg1* colonies generated extended filaments on serum and Spider plates, whereas *tup1* colonies formed truncated extensions (Figure 4B). Therefore, unlike *tup1* cells, *nrg1* cells are able to form normal hyphal cells under a range of experimental conditions.

The *tup1* and *nrg1* mutants displayed other phenotypic differences. Unlike the *tup1* strain, the *nrg1* mutant grew at 42°C (Figure 4A), grew on low glucose media and formed true hyphae on glycerol (not shown). Also, chlamydospore formation was delayed in the *tup1* mutant, unlike the *nrg1* strain (Figure 4B). Therefore, compared

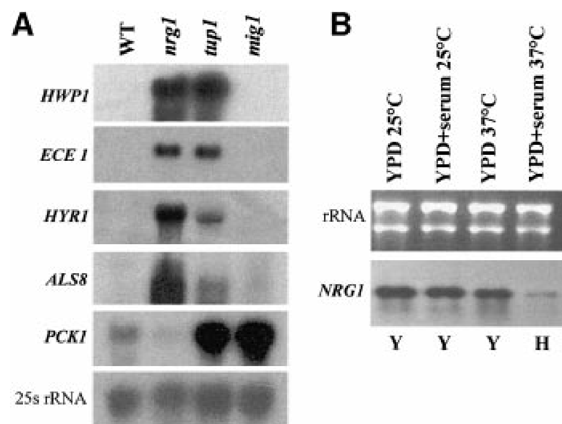

Fig. 5. Nrg1 represses hypha-specific genes. (**A**) Northern analysis of hypha-specific mRNAs (*HWP1*, *ECE1*, *HYR1* and *ALS8*) and a gluconeogenic mRNA (*PCK1*): WT, CAF2-1 (wild-type); *nrg1*, MMC3; *tup1*, BCa2-10; *mig1*, LOZ124 (Table I). (**B**) Northern analysis of *NRG1* mRNA levels during hyphal development in CAF2-1: Y, yeast form; H, hyphal form.

with CaNrg1, CaTup1 appears to play additional roles in the growth and development of *C.albicans*.

Expression of hypha-specific genes in nrg1, mig1 and tup1 mutants

In addition to controlling yeast–hypha morphogenesis, CaTup1 represses the expression of hypha-specific genes (Sharkey *et al*., 1999; Braun and Johnson, 2000; Brown *et al*., 2000). Therefore, we compared the expression of hypha-specific genes in *nrg1*, *mig1* and *tup1* strains (Figure 5A). As expected, the hypha-specific *HYR1*, *ALS8*, *HWP1* and *ECE1* mRNAs were not expressed in wild-type and *mig1* cells under conditions that promote growth of yeast cells. Significantly, these mRNAs were derepressed in *nrg1* and *tup1* cells. Therefore, both CaNrg1 and CaTup1 repress these hypha-specific genes under yeast growth conditions.

We also analysed the glucose-repressed *PCK1* gene (Leuker *et al*., 1997). The *PCK1* mRNA remained at low levels in wild-type and *nrg1* cells, but was derepressed in *tup1* and *mig1* cells. Hence, both CaTup1 and CaMig1 repress the *C.albicans PCK1* gene. Clearly, CaNrg1 only represses a subset of CaTup1-regulated genes in *C.albicans*.

CaNRG1 expression during yeast–hypha morphogenesis

We examined *CaNRG1* expression during the yeast–hypha transition in wild-type cells. *CaNRG1* mRNA levels were reduced 10-fold when hyphal growth was stimulated with serum at 37°C for 2 h (Figure 5B). Hence, *CaNRG1* expression appears to decline in response to serum stimulation.

Identification of CaNrg1-, CaTup1- and CaMig1-regulated genes by transcript profiling

CaNrg1 and CaTup1 co-regulate the *HYR1*, *ALS8*, *HWP1* and *ECE1* genes, and CaMig1 and CaTup1 co-regulate the *PCK1* gene (Figure 5A). Hence, CaNrg1 and CaMig1 appear to regulate distinct subsets of CaTup1-regulated genes, in a similar fashion to their *S.cerevisiae* counterparts (Treitel and Carlson, 1995; Smith and Johnson, 2000). To examine this further, we performed transcript profiling using arrays carrying 2002 *C.albicans* genes representing about one-quarter of the estimated protein-coding genes in this fungus.

Replicate membranes were hybridized with cDNA from wild-type, *nrg1*, *mig1* and *tup1* strains grown under conditions that favour the yeast form (CAF2-1, MMC3, LOZ124 and BCA2-10, respectively; Figure 6A). Expression was detected for 1851 genes (91.9%). A significant proportion of genes were derepressed >3-fold in the *nrg1* (10.7%), *mig1* (10.9%) or *tup1* (9.9%) mutants. As expected, distinct subsets of CaNrg1–CaTup1-co-regulated genes and CaMig1–CaTup1-co-regulated genes were observed (Figure 6B). Furthermore, the hypha-specific genes *ALS3*, *ALS8*, *HWP1* and *ECE1* all appeared in the list of the top 25 CaNrg1–CaTup1-co-regulated genes (Table II). Hence, most of the previously defined hypha-specific genes fell into the subset of CaNrg1–CaTup1-co-regulated genes defined by transcript profiling. This suggests that these transcription factors play a major role in the establishment of morphogenetic expression patterns in *C.albicans*, and that some of the unknown genes on this list might execute hypha-specific functions.

Not all genes that were regulated by CaNrg1 were also regulated by CaTup1 (Figure 6B). Hence, CaNrg1 might execute additional regulatory functions in *C.albicans*. The same was true for CaMig1. Hence, we sought functional categories that were enriched in the subsets of *C.albicans* genes that are regulated by CaNrg1, CaTup1 or CaMig1. This task was complicated by the fact that the functions of only a small proportion of *C.albicans* genes have been defined experimentally. Hence, for the purposes of this experiment, we assumed that sequence homologues execute similar functions in *C.albicans* and *S.cerevisiae* (Figure 6C). Certain functional categories were under-represented in CaNrg1- and CaMig1-regulated genes, compared with the gene set as a whole. These included transcription, cell growth and division, and protein destination (not shown). Other functional categories, such as metabolism and transport facilitation, were over-represented in both CaNrg1- and CaMig1-regulated subsets (not shown). CaMig1-, but not CaNrg1-regulated genes, were enriched for the functional category energy. In contrast, cell rescue functions were enriched in CaNrg1-, but not CaMig1-regulated genes (Figure 6C), suggesting that CaNrg1 might play a role in the regulation of stress responses as well as yeast–hypha morphogenesis.

Inactivation of CaNrg1 attenuates virulence and resistance to some stresses

To test whether CaNrg1 influences stress responses in *C.albicans*, we measured the doubling times of *nrg1* cells following exposure to certain stresses. The *nrg1* strain grew more slowly in YPD at 30°C (MMC3 $T_d = 190 \pm 9$ min) than its isogenic parent (CAF2-1 $T_d = 108 \pm 4$ min), but was as resistant to 0.7 M NaCl and amino acid starvation as the wild-type strain. However, the *nrg1* cells showed increased sensitivity to H_2O_2 ($T_d = 285 \pm 11$ min), 5% ethanol ($T_d = 447 \pm 62$ min) or carbon limitation (0.2% glucose: $T_d = 530 \pm 24$ min). Therefore, as suggested by transcript profiling, CaNrg1 influences some stress responses.

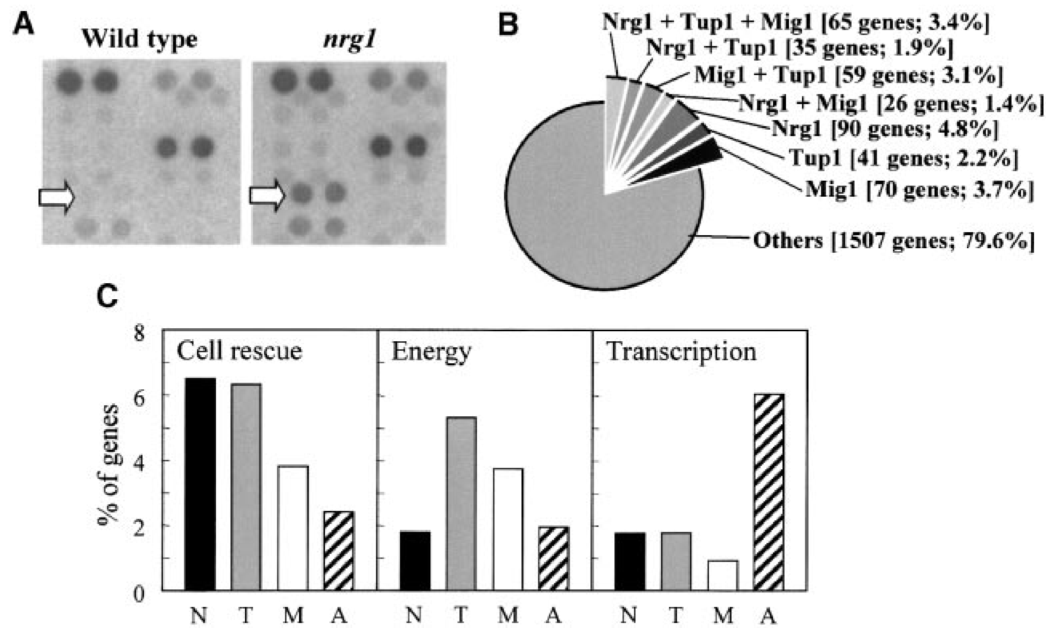

Fig. 6. The use of transcript profiling to identify targets of Nrg1 regulation. (**A**) ^{33}P signals from a small portion of membrane-based arrays carrying 2002 *C.albicans* ORFs, showing 28-fold derepression of one gene of unknown function in the *nrg1* strain (MMC3) compared with the wild-type control (CaF2). (**B**) Proportions of those genes that show detectable levels of expression, which are regulated by Nrg1, Tup1 and/or Mig1. (**C**) Percentage of regulated genes that are predicted to belong to the functional categories cell rescue, energy and transcription: Nrg1-regulated genes, N; Tup1-regulated genes, T; Mig1-regulated genes, M; all genes with a predicted function, A.

Table II. Top 25 Nrg1–Tup1-co-regulated *C.albicans* genes revealed by transcript profiling

Coordinate		Gene name[a]	Fold regulation[b]		Gene function	NREs[c]
			Nrg1	Tup1		
1	K24	*CaHWP1*	36.37	54.26	hyphal-specific cell wall protein	2
2	I9	orf6.6198	27.92	4.04	unknown function	1
1	L2	*CaALS3*	20.24	15.91	agglutinin-like cell surface glycoprotein	2
1	P14	orf6.7889	14.93	3.88	unknown function	1
1	B10	*CaALS8*	12.78	6.57	agglutinin-like cell surface glycoprotein	2
4	K9	*CaDDR48*	12.10	17.61	stress protein possibly involved in cell wall maintenance	2
2	J17	*CaCFL2*	11.75	61.51	ferric reductase	2
1	J1	*CaSAP5*	9.59	4.95	secreted aspartyl protease	1
3	O18	orf6.3649	7.95	4.14	unknown function	0
2	D22	*CaCRH1*	7.45	3.34	cell wall protein	0
4	I21	*CaCAN2*	7.41	5.72	*CAN1*-like amino acid permease	1
4	O9	*CaFTR1*	6.94	16.07	high-affinity iron permease	3
3	N16	orf6.686	6.06	3.28	unknown function	2
1	G12	*CaCAN1*	5.68	3.54	high-affinity arginine, lysine and histidine permease	2
5	J18	*CaGRE2*	5.66	7.19	reductase	1
5	P15	*CaRHR2*	5.24	7.28	DL-glycerol phosphate phosphatase	0
1	N2	*CaCEK1*	4.85	14.55	mitogen-activated protein kinase	0
2	F14	orf6.3066	4.80	5.08	unknown function	2
5	P22	orf6.6706	4.63	5.92	unknown function	3
3	L1	orf6.5333	4.48	5.45	unknown function	2
1	M1	*CaEXG1*	4.41	4.00	glucan 1,3-β-glucosidase involved in cell wall metabolism	1
1	N17	*CaECE1*	4.33	58.06	protein associated with hyphal growth	2
1	N1	*CaCBP1*	4.06	4.38	corticosteroid-binding protein	0
4	A1	*CaQDR1*	4.01	3.33	putative transporter involved in antibiotic resistance	2
2	C20	*CaPLC2*	3.78	3.00	phosphatidylinositol phospholipase C	0

[a]The Stanford annotation from sequence assembly 6 is provided for ORFs of unknown function (ftp://cycle.stanford.edu/pub/projects/candida/Ca-Assembly6.orf.gz).
[b]Fold regulation for a specific gene = (normalized mean signal in the mutant)/(normalized mean signal in the wild-type). Nrg1–Tup1-co-regulated genes were defined as genes that displayed >3-fold derepression in the *nrg1/nrg1* and *tup1/tup1* mutants, but <3-fold derepression in the *mig1/mig1* mutant.
[c]The numbers of NRE sequences [(A/C)(A/C/G)C_3T] within 1000 bp of the start codon are indicated.

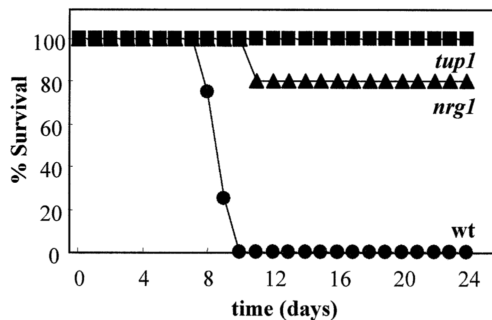

Fig. 7. Inactivation of CaNrg1 or CaTup1 attenuates the virulence of *C.albicans* in systemic infections. Survival of mice was monitored following injection of *C.albicans* cells into the tail vein: wild-type (CAF2-1, circles); *nrg1/nrg1* (MMC3, triangles); *tup1/tup1* (BCa2-10, squares).

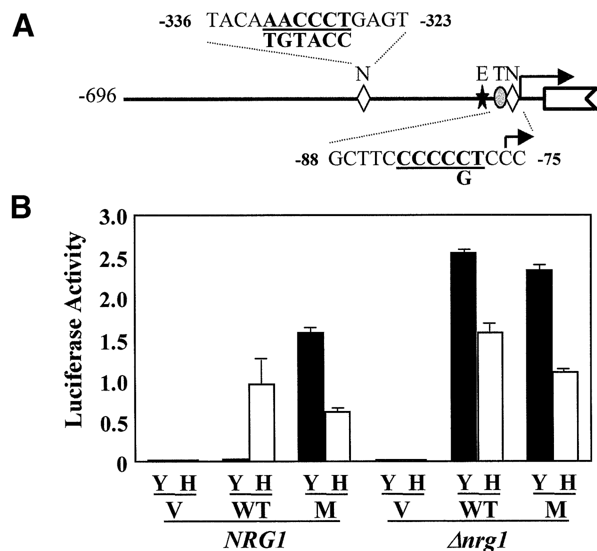

Fig. 8. Nrg1 response elements are required for Nrg1p-mediated repression. (**A**) Cartoon of the *ALS8* promoter showing the positions of NREs (N), an E-box (E; Leng *et al.*, 2001) and the TATA-box (T) relative to the major transcriptional start site. NRE sequences are highlighted (bold, underlined), and the mutations are show below the wild-type sequences. (**B**) Activities of wild-type and mutated *ALS8* promoter fusions in yeast (Y; closed bars; YPD at 25°C) and hyphal cells (H; open bars; YPD containing 10% serum at 37°C). Luciferase activities in CAI8 (*NRG1/NRG1*) and MMC9 (*nrg1/nrg1*) cells were measured for the empty *RrLUC* vector (pCRW3; V), the wild-type *ALS8* promoter (WT) and the mutated *ALS8* promoter (M).

CaNrg1–CaTup1-responsive genes included hypha-specific genes and other virulence-related functions such as a secreted aspartyl proteinase, adhesins and proteins involved in iron assimilation (Table II). Therefore, we compared the lethality of isogenic *C.albicans nrg1*, *tup1* and wild-type strains in a mouse model of systemic candidosis (Figure 7). The virulence of both mutants was attenuated significantly. The fungal load in the kidneys of infected animals was significantly lower for the *nrg1* (2.5×10^5 c.f.u./g) and *tup1* strains ($<1 \times 10^3$ c.f.u./g) compared with the parental strain (1.1×10^6 c.f.u./g). Hence, reduced fungal loads correlated with attenuated virulence. These data are consistent with the idea that CaNrg1 and CaTup1 regulate virulence traits.

Nrg1 response elements in C.albicans

CaNrg1 binds a C_4T-containing sequence *in vitro* (Figure 2), and ScNrg1 interacts with C_4T or C_3TC (Park *et al.*, 1999). Hence, we searched for these elements in the top 25 CaNrg1–CaTup1-co-regulated promoters. Some CaNrg1-regulated genes, e.g. *ECE1* (Figure 5), lacked these elements. Therefore, the sequences C_4T and C_3TC are not sufficient to define an Nrg1 response element (NRE) in *C.albicans*.

Further *in silico* analyses of CaNrg1–CaTup1-responsive promoters using regulatory sequence analysis tools (van Helden *et al.*, 2000) revealed that most carry an element that is closely related to the core sequence C_3T: (A/C)(A/C/G)C_3T (Table II). The *ALS8* promoter, which is regulated by CaNrg1 (Figure 5), carries two such sequences (Figure 8A). Hence, we tested whether *ALS8* repression is dependent upon these putative NREs using the *Renilla reniformis* luciferase reporter (Srikantha *et al.*, 1996). As expected, an *ALS8-RrLUC* reporter was derepressed by inactivation of CaNrg1 (Figure 8B). However, mutation of the C_4TC region alone at –80 did not derepress the *ALS8* promoter in yeast cells (not shown), confirming that C_4T and C_3TC are not sufficient to define the *C.albicans* NRE. Inactivation of both putative NREs did relieve *ALS8-RrLUC* repression in wild-type *CaNRG1* cells growing in the yeast form (Figure 8B). The level of derepression of this mutant *ALS8* promoter was not increased further in *nrg1* cells, indicating that these effects were not additive. Furthermore, the levels of derepression were equivalent if not greater than those observed for the *ALS8* promoter in wild-type hyphae (Figure 8B). Therefore, these (A/C)(A/C/G)C_3T sequences mediate the repression of *ALS8* by CaNrg1 during growth in the yeast form, and this repression is released completely by inactivation of CaNrg1 alone.

Discussion

Mode of action of CaNrg1

We identified the *C.albicans NRG1* gene on the basis that CaNrg1 can bind specifically to C_4T-containing oligonucleotides. This sequence-specific DNA binding activity was confirmed using gel shift assays (Figure 2). DNA sequencing revealed that *S.cerevisiae* Nrg1 is the closest known homologue of CaNrg1 (Figure 1). Although ScNrg1 has been reported to interact with C_4T or C_3TC (Park *et al.*, 1999), two observations suggest that these elements are not sufficient to account for CaNrg1 binding at *C.albicans* promoters. First, the *ECE1* promoter lacks C_4T or C_3TC, but is strongly repressed by CaNrg1 (Figure 5). Secondly, mutation of the single C_4TC sequence in the *ALS8* promoter does not relieve its repression by CaNrg1. Detailed *in silico* analyses revealed a related sequence, (A/C)(A/C/G)C_3T, which is present in most of the promoters of CaNrg1–CaTup1-regulated genes (Table II). The genes in Table II that do not carry this element may be regulated indirectly by CaNrg1. Significantly, two of these elements exist in the *ALS8* promoter, and CaNrg1-mediated repression of this promoter is relieved when both of these elements are mutated (Figure 8). Therefore, CaNrg1 is a sequence-specific

DNA-binding protein that represses transcription via (A/C)(A/C/G)C_3T. We have called this sequence the NRE.

ScNrg1 interacts physically with ScTup1 complexes (Park *et al*., 1999). By analogy with *S.cerevisiae*, CaNrg1 might interact with a CaTup1 complex to regulate gene expression in *C.albicans*. The fact that *C.albicans nrg1* and *tup1* mutants display related phenotypes is consistent with this idea. Both *nrg1* and *tup1* mutants show constitutive filamentous morphologies, wrinkly colonies and invasive growth (Figures 3 and 4; Braun and Johnson, 1997). Also, hypha-specific genes are derepressed in both *nrg1* and *tup1* mutants (Figure 5). Furthermore, transcript profiling confirms that a subset of *C.albicans* genes is co-regulated by CaNrg1 and CaTup1 (Figure 6). Hence, CaNrg1 might target CaTup1 to a subset of *C.albicans* promoters. However, our data suggest that CaNrg1 does more than target CaTup1 to hypha-specific promoters, because some CaNrg1-regulated genes are not derepressed in the *tup1* mutant (Figure 6).

CaNrg1, CaTup1 and CaMig1 regulate distinct but overlapping subsets of genes

In *S.cerevisiae*, ScTup1 regulates the expression of several distinct sets of genes, only some of which are controlled by ScNrg1 or ScMig1 (e.g. Keleher *et al*., 1992; Treitel and Carlson, 1995; Park *et al*., 1999; Smith and Johnson, 2000). By analogy, CaNrg1 and CaMig1 might control the expression of different subsets of CaTup1-regulated genes in *C.albicans*. As predicted, the hypha-specific genes *ALS8*, *ECE1*, *HWP1* and *HYR1* were co-regulated by CaNrg1 and CaTup1, but not by CaMig1 (Figure 5). Also, *PCK1* was co-regulated by CaMig1 and CaTup1, but not by CaNrg1. The more global view provided by transcript profiling showed clearly that CaNrg1, CaMig1 and CaTup1 repress distinct, but overlapping gene sets in *C.albicans* (Figure 6). However, some genes were co-regulated by all three factors, and some genes were only controlled by one of these three factors.

These data indicate that CaNrg1 and CaTup1 play overlapping but distinct roles in *C.albicans*, and hence that *nrg1* and *tup1* mutants might display phenotypic differences. As predicted, such differences were observed. The *nrg1* cells form hyphae following serum induction, whereas *tup1* cells appear to be fixed in a pseudohyphal state (Figure 3). This is reflected in the different behaviour of *nrg1* and *tup1* colonies on a range of solid media (Figure 4). Further subtle differences between the mutants were observed. Unlike *tup1* cells, *nrg1* cells are able to grow following carbon limitation or at 42°C (Figure 4). Also, chlamydospore formation is delayed in the *tup1* strain, but not in the *nrg1* mutant (Figure 4).

These subtle differences between *nrg1* and *tup1* cells may be due to the pleiotropic nature of the *tup1* mutation. In other words, the inability of *tup1* cells to form hyphae might be due to secondary effects of the mutation caused by changes in metabolism, for example. Pleiotropic effects of the *tup1* mutation might also contribute to the difficulties in defining the hierarchical relationships between CaTup1 and other morphological regulators (Braun and Johnson, 2000).

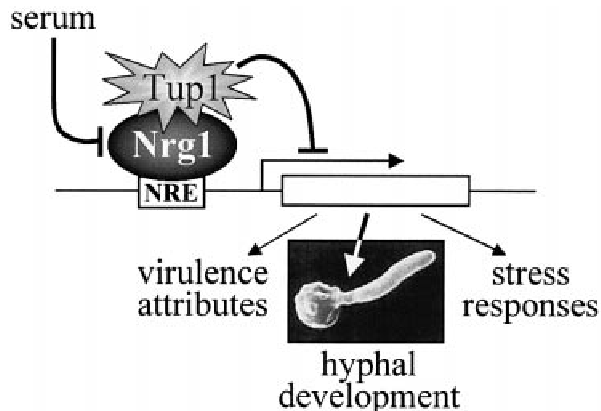

Fig. 9. Model for Nrg1 function in *C.albicans*. According to this model, CaNrg1 binds to the NRE and brings the CaTup1 repressor to the promoters of genes involved in hyphal development, some stress responses and virulence attributes. *CaNRG1* mRNA levels are down-regulated by serum, suggesting that morphogenetic signals can relieve CaNrg1-mediated repression by reducing CaNrg1 levels. CaNrg1 also appears to execute CaTup1-independent roles in *C.albicans* (see text).

Biological roles of CaNrg1

Several observations indicate that CaNrg1 plays a central role in the negative regulation of yeast–hypha morphogenesis (Figure 9). Most significantly, the inactivation of CaNrg1 derepressed filamentous and invasive growth (Figures 3 and 4), and derepressed hypha-specific genes (Figure 5; Table II). The repression of hypha-specific genes by CaNrg1 appears to be direct, because these genes contain at least one NRE in their promoter (Figure 8; Table II). However, CaNrg1 might also play an indirect role in the control of hyphal development, because *CEK1* was present in the CaNrg1–CaTup1-co-regulated gene set revealed by transcript profiling (Table II). *CEK1* encodes the MAPK on one of the morphogenetic signalling pathways that activates hyphal development (Csank *et al*., 1998; Brown and Gow, 1999; Ernst, 2000). Therefore, CaNrg1 might regulate hyphal development at two levels: directly by repressing hypha-specific functions, and indirectly by down-regulating the MAPK signalling pathway. This repression appears to be relieved, at least in part, by the down-regulation of *CaNRG1* in response to a morphogenetic signal (Figure 9).

A second putative CaTup1-targeting protein regulates morphogenesis in *C.albicans*. Inactivation of Rfg1, a homologue of *S.cerevisiae* Rox1, derepresses hyphal growth and hypha-specific genes (Kadosh and Johnson, 2001). Hence, some *rfg1* and *nrg1* phenotypes are similar. However, *nrg1* cells seem to display stronger hyphal phenotypes than *rfg1* cells (Braun *et al*., 2001). Also, CaNrg1-mediated repression is sufficient to account for the repression of at least one hypha-specific gene (Figure 8). Nevertheless, in the absence of a morphogenetic stimulus, *nrg1* cells grow as pseudohyphae (Figure 4), suggesting that inactivation of CaNrg1 does not fully derepress hyphal development. Hence, CaNrg1 and Rfg1 both repress morphogenesis, apparently via similar mechanisms (Figure 9; Braun *et al*., 2001; Kadosh and Johnson, 2001), although CaNrg1 might play the predominant role.

CaNrg1 plays an additional role in some stress responses (Figure 6). The NRE (A/C)(A/C/G)C_3T includes the sequence C_4T, which acts as a stress response element (STRE) in *S.cerevisiae* (Marchler *et al*., 1993; Mager and De Kruijff, 1995). The sequence C_4T is present in the promoters of stress response genes in *C.albicans* (not shown), and putative cell rescue functions are over-represented in the subset of CaNrg1-regulated genes revealed by transcript profiling (Figure 6). However, not all (A/C)(A/C/G)C_3T sequences contain C_4T. Hence, there is only partial overlap between NREs and STREs. Not surprisingly, therefore, *C.albicans nrg1* mutants only displayed increased sensitivity to a subset of stresses, including an oxidative stress, ethanol treatment and carbon limitation. Nevertheless, CaNrg1 provides a mechanistic link between stress responses and cellular morphogenesis in *C.albicans* (Brown and Gow, 1999).

Our data also provide clues about the role of CaMig1 in *C.albicans*. Consistent with the findings of Zaragoza *et al*. (2000), the *C.albicans mig1* mutant did not display a morphological phenotype, except on Spider medium (Figure 4). Also, CaMig1 does not influence the expression of hypha-specific genes (Figure 5). However, CaMig1 does regulate the gluconeogenic gene *PCK1* (Figure 5), and putative energy functions were over-represented in the subset of CaMig1-regulated genes (Figure 6), suggesting that CaMig1 might control aspects of *C.albicans* metabolism, like its homologue in *S.cerevisiae* (Gancedo, 1998).

Co-regulation of virulence factors by CaNrg1 and CaTup1

Our data also provide evidence for the co-regulation of virulence factors in *C.albicans*. Several reports have suggested that the expression of some virulence factors, such as adhesion and morphogenesis, might be linked in *C.albicans* (Hube *et al*., 1994; Staab *et al*., 1996, 1999; Leng, 1999). Our data reinforce these observations, since the set of CaNrg1–CaTup1-co-regulated genes includes adhesins (*HWP1*, *ALS3* and *ALS8*), a secreted aspartyl proteinase (*SAP5*) and two genes involved in iron assimilation (*CFL1* and *FTR1*; Yamada-Okabe *et al*., 1996; Ramanan and Wang, 2000) (Table II). Hence, CaNrg1 might play a central role in the coordination of key virulence attributes in *C.albicans* (Figure 9). Not surprisingly, therefore, inactivation of CaNrg1 significantly attenuates the virulence of *C.albicans* (Figure 8).

Materials and methods

Strains and growth conditions

Candida albicans strains (Table I) were grown in: YPD (Sherman, 1991); YPD containing 10% fetal calf serum; Soll's medium at pH 4.5 or 6.5 (Swoboda *et al*., 1994); SC, synthetic complete medium (Kaiser *et al*., 1994); YNB medium containing low glucose (0.67% yeast nitrogen base without amino acids, 0.2% glucose); SLAD agar (Gimeno *et al*., 1992); Spider agar (Liu *et al*., 1994); and cornmeal–Tween agar (Braun and Johnson, 1997). The *C.albicans* cell morphology was analysed using an Olympus BX50 microscope mounted with a 35 mm Olympus camera, and quantified using an improved Neubauer haemocytometer. Colonies were analysed using a Zeiss stereo microscope stemi 2000-C.

CaNRG1 isolation

A *C.albicans* cDNA expression library in λ-ZAPII (Swoboda *et al*., 1993) was screened using published procedures (Singh, 1993) with a C_4T-containing oligonucleotide (top strand, 5′-CGCGTGTATAAAC**CCCCT**TTTCTTGGGG**CCCCT**TTTCTTGGGGGA). Clones that gave positive and reproducible signals with this oligonucleotide, but no significant signals with oligonucleotides lacking C_4T, were selected. A cDNA clone was converted into its pBlueScript form (Stratagene, Cambridge, UK) to create pBS-NRG1, and was then used to isolate the complete *NRG1* locus from a *C.albicans* genomic library (Smith *et al*., 1992) by colony hybridization (Sambrook *et al*., 1989).

CaNRG1 disruption

The *NRG1* locus (–1262 to +1243) was cloned into pGEM-T® (Promega, Southampton, UK). Reverse PCR was used to mutate the initiation codon to GTG, delete codons 92–237 and introduce a *Bgl*II site at the deletion to generate pGEM-Δnrg1. The 3 kb *hisG-URA3-hisG* sequence (Fonzi and Irwin, 1993) was then inserted into pGEM-Δnrg1 to create the *nrg1::hisG-URA3-hisG* cassette. This cassette was released from the pGEM-T backbone using *Not*I and transformed into *C.albicans* (Gietz and Woods, 1998). Both *CaNRG1* alleles in the strains CAI4 and CAI8 (Table I) were disrupted using two rounds of ura-blasting (Fonzi and Irwin, 1993). Disruptions were confirmed by Southern blotting and PCR diagnosis.

To create *MET3-NRG1*, the *NRG1* open reading frame (ORF) was PCR amplified using the primers 5′-CATTAAGATCTAAACAATCATTATGC and 5′-GCAATTAACCCTCGAGATTTAACCCG (*Bgl*II and *Xho*I sites underlined), cloned into pGEM®-T Easy to make pGEM-NRG1, and resequenced. The *NRG1* ORF was then released from pGEM-NRG1 using *Pst*I and *Sph*I, and ligated into pCaEXPa (Care *et al*., 1999) to make pMET3-NRG1. This plasmid was linearized with *Stu*I, transformed into *C.albicans*, and single copy integration at the *RP10* locus (Murad *et al*., 2000) was confirmed by Southern blotting.

RNA and DNA analyses

Published methods were used for RNA and DNA preparation, Southern blotting and northern analysis (Hoffman and Winston, 1987; Brown, 1994; Wicksteed *et al*., 1994; Planta *et al*., 1999). cDNA and genomic clones were sequenced using Big Dye Terminator Cycle Sequencing kits (Perkin Elmer, Workington, UK) and run on an ABI 377 automated DNA sequencer. The *NRG1* sequence has DDBJ/EMBL/GenBank accession No. AF321521, and is available in the Stanford University *C.albicans* genome sequence, in Contig6-2506 (http://www-sequence.stanford.edu/group/candida/search.html). DNA sequences were analysed at the Stanford Genome Database (http://genome-www.stanford.edu/), and promoter sequences were analysed using regulatory sequence analysis tools (http://www.ucmb.ulb.ac.be/bioinformatics/rsa-tools/; van Helden *et al*., 2000).

Transcript profiling

Details of the transcript profiling methods will be published elsewhere (A.M.A.Murad, C.Gaillardin, C.d'Enfert, F.Tekaia, D.Marechal, D.Talibi and A.J.P.Brown, submitted). Briefly, ORFs were identified using Release 3 of the *C.albicans* genome sequence obtained from the Stanford DNA Sequencing and Technology Center website at http://www-sequence.stanford.edu/group/candida (April 1999). Replicate arrays of PCR products corresponding to 2002 *C.albicans* ORFs were hybridized with [^{33}P]cDNA prepared using RNA from mid-exponential *C.albicans* cells (Hauser *et al*., 1998). Cells were grown in YPD at 30°C to an OD_{600} of 0.8, subcultured into fresh YPD at 30°C, grown to an OD_{600} of 0.8 and then harvested for analysis. Signals were detected using a Fuji FLA-3000 phosphoimager and analysed using Array Vision software (Amersham, Buckinghamshire, UK).

Gel shift assays

Synthetic Nrg1 was made *in vitro* using pBS-NRG1 and the TNT® reticulocyte lysate coupled transcription/translation system (Promega, Southampton, UK). Control reactions were performed using the control luciferase DNA provided by the manufacturers. ^{35}S-labelled products were analysed by SDS–PAGE. Unlabelled reaction mixes were used in gel shift assays with ^{32}P-end-labelled double-stranded oligonucleotides (Sambrook *et al*., 1989): C_4T-containing oligonucleotide, 5′-GTCCTGTATAAAC**CCCCT**TTTCTTGGGG**CCCCT**TTTCTTGGGGGAG (top strand); control oligonucleotide, 5′-GTCCTGGCTTCCAAAAGACCTTGAATTGAGGTCTGATAGTAG (top strand). Assays were performed on 20 fmol of oligonucleotide with 20 ng of iv product and 0.5 μg/ml poly(dI–dC)·poly(dI–dC), as described (Carey, 1991), and electrophoresed on 5% polyacrylamide gels.

Reporter assays

RrLUC promoter fusions were made using pCRW3 (Srikantha *et al*., 1996). The wild-type *ALS8* promoter, site-directed from –696 to +4, was fused in-frame with the *RrLUC* ORF. Putative NREs were altered by mutagenesis and resequenced (Sambrook *et al*., 1989) as shown in Figure 8A. Wild-type and mutant *ALS8-RrLUC* plasmids and the pCRW3 control were linearized with *Hin*dIII, and transformed into *C.albicans* CAI8 (Gietz and Woods, 1998). Single copy integration at the *ade2* locus was confirmed by PCR diagnosis. To measure luciferase activities, protein extracts were prepared from *C.albicans* transformants after 3 h growth in YPD at 25°C, or YPD containing 10% serum at 37°C. Quadruplicate luciferase assays (in RLU/20 µg protein/20 s) were performed on fresh extracts with 0.5 µM co-elentrazine (Molecular Probes, Leiden, The Netherlands) in a Lumat LB9507 luminometer (EG&G Berthold) (Srikantha *et al*., 1996). Similar data were obtained in three experiments using independent transformants.

Virulence assays

The virulence of *C.albicans* strains was assessed in a mouse model of systemic candidosis. Cells were grown for 24 h at 30°C in 4% glucose, 1% peptone, 0.1% yeast extract, 1% glycerol, harvested by centrifugation, and washed twice with water. Fungal biomass was compared on the basis of ATP concentration (Odds and Abbott, 1984), and an equivalent of 5000 c.f.u. per mouse was injected into the lateral tail vein of DBA2 mice (average weight 19 g; Harlan UK).

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