Differential regulation of the transcriptional repressor NRG1 accounts for altered host cell interactions in *Candida albicans* and *Candida dubliniensis*

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Summary

*Candida dublieniensis* is genetically closely related to *C. albicans*, but causes fewer infections in humans and exhibits reduced virulence and filamentation in animal models of infection. We investigated the role of the *C. dublieniensis* transcriptional repressor-encoding gene *CdNRG1* in regulating this phenotype. Deletion of both copies of *CdNRG1* increased the formation of true hyphae by *C. dublieniensis* in response to serum, exogenous cAMP and CO₂. In addition, deletion of *CdNRG1* greatly enhanced filamentation and survival of *C. dublieniensis* in co-culture with murine macrophages. In the reconstituted human oral epithelium (RHE) infection model, the *nrg1Δ* mutant caused increased tissue damage relative to the wild-type strain. However, deletion of *CdNRG1* did not change the virulence of *C. dublieniensis* in the systemic mouse model of infection. The increased rate of hypha formation in *C. albicans* relative to *C. dublieniensis* in response to phagocytosis by macrophages and serum was associated with rapid downregulation of *NRG1* expression in *C. albicans*. This study demonstrates that the reduced virulence of *C. dublieniensis* is due to the inability of this species to modulate *NRG1* expression in response to the same environmental signals that promote filamentation in *C. albicans*. 
Introduction

*Candida dubliniensis* is closely related to the pathogenic yeast *Candida albicans* and shares many of its characteristic phenotypic traits, such as the ability to produce true hyphae and chlamydospores (Gilfillan et al., 1998; Sullivan et al., 1995). *Candida dubliniensis* was first described in 1995 and is mainly associated with oral candidosis in severely immunocompromised patients, including those with HIV-infection and AIDS (Sullivan et al., 1995; Sullivan et al., 2004). Although numerous studies have subsequently described the isolation of *C. dubliniensis* from various patient groups and anatomical sites, current evidence suggests that its prevalence in the human population is significantly lower than that of *C. albicans* (Sullivan et al., 2004). Strikingly, the incidence of haematogenous candidosis caused by *C. dubliniensis* in England and Wales was reported to be 30-fold lower than that caused by *C. albicans* (Kibbler et al., 2003). The results of similar epidemiological studies throughout the world confirm that *C. dubliniensis* is rarely responsible for systemic candidosis (Sullivan et al., 2004). This lower incidence of infection suggests that *C. dubliniensis* has a reduced ability to cause the type of infections normally associated with *C. albicans* (i.e. mucosal and systemic candidosis). Two studies have shown that mice infected with *C. dubliniensis* isolates via intravenous injection have significantly higher survival rates than those infected with a similar dose of *C. albicans* (Gilfillan et al., 1998; Vilela et al., 2002). Recently, Stokes et al. (2007) using an infant mouse oral intragastric model of infection showed that *C. dubliniensis* failed to colonise the gastric mucosa and was less successful than *C. albicans* at passing into the bloodstream to cause haematogenous infection. Furthermore, *C. dubliniensis* exhibited less filamentation *in vivo* compared to *C. albicans*, particularly following systemic spread to the kidneys, a phenotype also noted by Vilela et al. (2002).
Filamentation is now widely accepted to be one of the major virulence factors of *C. albicans*. *Candida dubliniensis* produces fewer hyphae than *C. albicans* under most environmental conditions that promote this morphological transition, including growth in serum and following shifts in pH and temperature (Gilfillan *et al.*, 1998; Stokes *et al.*, 2007). In addition, a recent survey of the *C. dubliniensis* genome using DNA microarrays revealed that several hypha-specific virulence-associated genes of *C. albicans* were either absent in *C. dubliniensis* (e.g. the proteinases SAP5 and SAP6) or were significantly divergent at the nucleotide sequence level (e.g. the hypha-specific adhesin *HWP1*) (Moran *et al.*, 2004). These findings suggest that *C. albicans* hyphal structures may be better adapted to colonise or infect certain tissues *in vivo*. We hypothesise that this reduced filamentation phenotype may be one of the major reasons why *C. dubliniensis* is less invasive and, therefore, less pathogenic than *C. albicans*.

Despite these findings, there are specific environmental conditions in which *C. dubliniensis* produces filaments and chlamydomspores more efficiently than *C. albicans*, such as on Staib (Niger [*Guizotia abyssinica*] seed creatinine) agar, and on tobacco extract-based media and Pal’s agar (Al-Mosaid *et al.*, 2003; Khan *et al.*, 2004; Staib and Morschhäuser, 1999). Staib & Morschhäuser (2005) recently identified the molecular basis for this phenotypic difference. They showed that a zinc-finger DNA-binding protein, Nrg1p, is differentially expressed in the two species when grown in Staib medium. The *CaNRG1* gene is a homologue of the *Saccharomyces cerevisiae NRG1* gene encoding a transcriptional repressor and was first identified in *C. albicans* as a repressor of filamentation (Braun *et al.*, 2001). *Candida albicans* Nrg1p is a sequence-specific DNA-binding protein that targets the Ssn6-Tup1 co-repressor complex to a subset of genes that are transcriptionally
activated during hypha formation. This program of Tup1p-mediated transcriptional repression is controlled at least in part by regulation of the expression of NRG1 transcription (Murad et al., 2001a; Murad et al., 2001b). Staib & Morschhäuser (2005) showed that the C. dubliniensis NRG1 gene is specifically downregulated on Staib medium, thus allowing formation of hyphae and chlamydospires on this medium. In contrast, C. albicans NRG1 is constitutively expressed in Staib medium and cells remain in the budding yeast phase of growth.

In the present study, we set out to examine the role of hypha formation as a determinant of differential virulence in C. albicans and C. dubliniensis. We examined the filamentation and virulence of both species during infection of the murine macrophage cell line RAW264.7, and identified the effects of deletion of CdNRG1 in C. dubliniensis on filamentation and virulence in this model and also during infection of the reconstituted human oral epithelial (RHE) cell model and following intravenous inoculation in mice (Marcil et al., 2002; Schaller et al., 1998).
Results

Candida dubliniensis does not form true hyphae following phagocytosis by RAW264.7 macrophages

Several studies have demonstrated that under many in vitro and in vivo conditions *C. dubliniensis* forms hyphae at a reduced rate compared to *C. albicans* (Gilfillan *et al.*, 1998; Stokes *et al.*, 2007). In order to study the effects of reduced filamentation in *C. dubliniensis* on virulence, we used a simple *Candida*-macrophage co-culture model with the murine cell line RAW264.7. Several investigators have demonstrated that filamentation is crucial to survival and proliferation of *C. albicans* during co-culture with murine macrophage cells (Lo *et al.*, 1997; Lorenz and Fink, 2001; Marcil *et al.*, 2002). Infection of monolayers of RAW264.7 macrophages (1.5 x 10⁵ cells) with *C. albicans* or *C. dubliniensis* yeast cells at multiplicities of infection (MOIs) of 1:2 to 1:128 (*Candida*:macrophage) demonstrated that >90% of *Candida* cells of both species were phagocytosed by 1 h. However, within 3 h of inoculation, phagocytosed *C. albicans* cells had begun to form hyphae, which protrude from within the macrophage (Fig. 1A). In contrast, *C. dubliniensis* cells failed to form true hyphae following phagocytosis and failed to escape from the phagolysosome (Fig. 1B). Following a 16 h co-culture with these macrophage cells at MOIs of 1:2 to 1:128, the proliferation of *C. dubliniensis* isolates was greatly reduced relative to macrophage-free growth controls containing the same inoculum (Fig. 1C). The growth of *C. albicans* isolates in macrophage co-culture was significantly greater (p < 0.001) compared to growth of *C. dubliniensis* in macrophage co-culture at all MOIs except 1:128 (Fig. 1C).
Stimulation of the RAS-cAMP pathway does not promote filamentation in C. dubliniensis

In order to investigate the molecular basis for the significantly reduced filamentation in *C. dubliniensis* relative to *C. albicans*, we examined whether stimulation of the RAS1-cAMP pathway could induce the formation of true hyphae. Preliminary bioinformatics analysis of the *C. dubliniensis* genome ([http://www.sanger.ac.uk/sequencing/Candida/dubliniensis/](http://www.sanger.ac.uk/sequencing/Candida/dubliniensis/)) suggests that the genes encoding the major elements of this pathway are highly conserved in *C. dubliniensis* (data not shown). In particular, *C. dubliniensis RAS1* shares 83% identity at the amino acid sequence level with its *C. albicans* orthologue. We attempted to stimulate the Ras1p-activated cAMP signalling pathway in *C. dubliniensis* by using a dominant active *CaRAS1* allele containing the G13V substitution which has been shown to render Ras1p hyperactive in *C. albicans* and *S. cerevisiae*. We transformed *C. dubliniensis* CDUM4B with the ARS-containing plasmid pLJ65, which contains a hyperactive *RAS1* allele (*RAS1*<sup>G13V</sup>) under the control of the *C. albicans PCK1* promoter (Leberer *et al.*, 2001). In our hands, this promoter could drive expression of pGFP3 in *C. albicans* and *C. dubliniensis* when grown in medium containing 1% (w/v) casamino acids as the sole carbon source (data not shown). Transformation of *C. albicans* CAI4 with the *RASI*<sup>G13V</sup> expression plasmid (pLJ65) yielded highly wrinkled colonies consisting of filamentous cells following growth on selective medium containing 1% (w/v) casamino acids at 30°C. However, in *C. dubliniensis* CDUM4B, expression of *RASI*<sup>G13V</sup> did not result in any significant change in colonial morphology when compared with strains harbouring the empty pVEC vector (data not shown). In liquid culture, when transformants of both species were grown at 30°C in minimal medium containing 2% (w/v) glucose and transferred to medium containing
1% (w/v) casamino acids, both species initially produced germ-tubes, probably due to changes in carbon source and cell density. However, following 6 h incubation, *C. albicans* harbouring pLJ65 became hyperfilamentous compared to transformants harbouring the empty pVEC vector (Fig. 2A). In contrast, *C. dubliniensis* transformants harbouring pVEC or pLJ65 reverted to budding or pseudohyphal growth (Fig. 2A). Also, the addition of cAMP (10 mM dibutyryl-cAMP) to liquid DMEM medium failed to stimulate production of filaments in *C. dubliniensis* Wü284, whereas addition of cAMP to *C. albicans* SC5314 cells accelerated hypha production (Fig. 2B). These data suggested that activation of filamentation via the Ras1p-cAMP pathway may be under strong negative repression in *C. dubliniensis*.

Deletion of *C. dubliniensis* NRG1 increases the rate of filamentation

Recently, Staib & Morschhäuser (2005) described a homologue of the *C. albicans* filamentation repressor-encoding gene *NRG1* in *C. dubliniensis*, encoding a protein with 86% similarity at the amino acid sequence level to *C. albicans* Nrg1p. We deleted both copies of the *NRG1* gene in the *C. dubliniensis* strain Wü284 using the SAT1-flipper technique, generating the homozygous CdNRG1 deletion strain CDM10 (Reuss et al., 2004) (Fig. 3). On normal YPD agar at 37°C, this strain produced wrinkled colonies consisting of elongated yeast cells and pseudohyphae, whereas the wild-type strain formed smooth colonies (Fig. 4A). The mutant also formed highly wrinkled colonies with hyphal fringes on Spider medium and serum-containing medium (Fig. 4A). Introduction of either the *C. albicans* or *C. dubliniensis* NRG1 genes under control of their own promoters on an integrative plasmid could restore the wild-type smooth colony phenotype on most media (strains CDM11 and CDM12, Fig. 4A). However, the phenotype on Spider medium was only partially
complemented by introducing a single copy of \textit{CdNRG1} in strain CDM11, as sectors of the colony exhibited irregular edges (Fig. 4A) On Staib medium, wild-type \textit{C. dubliniensis} strains have been shown to downregulate \textit{NRG1}, yielding rough colonies with hyphal fringes. The \textit{nrg1\Delta/nrg1\Delta} mutant CDM10 also produced this phenotype, as did the \textit{C. albicans} and \textit{C. dubliniensis} \textit{NRG1} reintegrant strains CDM11 and CDM12. These data indicate that trans-acting factors in \textit{C. dubliniensis} can downregulate both the \textit{CaNRG1} and \textit{CdNRG1} genes in \textit{C. dubliniensis} when grown on Staib agar. In liquid DMEM medium at 37°C, wild-type \textit{C. dubliniensis} cells grew as yeasts and pseudohyphae. The \textit{C. dubliniensis nrg1\Delta/nrg1\Delta} mutant CDM10 was predominantly pseudohyphal in this medium, but unlike the wild-type strain, formation of true hyphae could be stimulated by the addition of 10 mM dibutyryl-cAMP or by growth in the presence of 5% (v/v) CO\textsubscript{2} (Fig. 4B). Again, the non-filamentous wild-type phenotype could be restored by introduction of either the \textit{C. albicans} or \textit{C. dubliniensis} \textit{NRG1} genes. We also compared the rate of true hypha formation in water containing 50 % (v/v) foetal bovine serum (Fig. 4C). The wild-type \textit{C. dubliniensis} strain Wü284 produced true hyphae under these conditions, but the rate of formation was reproducibly higher in the \textit{nrg1\Delta/nrg1\Delta} mutant strain CDM10. The growth rate of the mutant strain in YPD broth at 37°C was essentially identical to that of the wild-type strain (doubling times of 75.5 min and 76.5 min, respectively). CDM10 did not exhibit increased tolerance to environmental stress, and like the wild-type failed to grow at 42°C and grew slowly on medium containing 1 M NaCl. We did not observe any difference in susceptibility to oxidative stress tested by growing CDM10 and wild-type cells in H\textsubscript{2}O\textsubscript{2} or the nitric oxide-generating molecule dipropylentriamine NONOate (Hromatka \textit{et al.}, 2005).
Differential expression of NRG1 in C. dubliniensis and C. albicans

Staib & Morschhäuser (2005) showed that the NRG1 gene is differentially regulated in *C. albicans* and *C. dubliniensis* when cultured on Staib medium (syn. *Guizotia abyssinica* creatinine agar). On this medium, *C. dubliniensis* produces filaments due to downregulation of the NRG1 gene. We hypothesised that the opposite may be true under conditions when *C. albicans* encounters host cells (e.g. macrophages), or when conditions are similar to those encountered *in vivo* (e.g. serum at 37°C), that is, that NRG1 mRNA levels may be downregulated more rapidly in *C. albicans* in response to host signals. Using the yGFP3 coding sequence (Cormack *et al.*, 1997) fused to the *C. albicans* and *C. dubliniensis* NRG1 promoter sequences, we could monitor the activity of this promoter under a variety of experimental conditions. This reporter gave results similar to those reported by Staib & Morschhäuser (2005) in liquid Staib medium in which *C. dubliniensis* formed chlamydospores. Fluorescence of *C. dubliniensis* chlamydospores produced in this medium was greatly reduced compared to yeast cells (data not shown). The activity of the NRG1 promoter was also monitored during phagocytosis of yeast cells from both species by RAW264.7 macrophages. By 5 h post inoculation of the macrophages, cells of *C. dubliniensis* were still within the phagolysosome and exhibited strong green fluorescence (Fig. 5A). In contrast, *C. albicans* cells were initially fluorescent following phagocytosis, with fluorescence greatly reduced in hyphae following 5 h co-culture (Fig. 5A). By using real-time PCR we directly quantified the level of NRG1 expression in both species relative to expression of TEF1 transcript. Following 1 h co-culture with macrophages, expression levels of NRG1 in *C. albicans* had dropped to less than 15% of that in preculture cells in YEPD. This decrease coincided with the production of true hyphae of *C. albicans* under these conditions. However, in *C. dubliniensis*,
although expression levels initially dropped to approximately 50% of preculture levels, expression then increased to ~70% of that observed in preculture cells (Fig. 5B). In 10% (v/v) foetal calf serum, both species produce true hyphae; however, *C. albicans* produces hyphae more rapidly than *C. dubliniensis*. When NRG1 expression levels were compared under these conditions, we observed a much more rapid repression of NRG1 expression in *C. albicans* than in *C. dubliniensis* (Fig. 5C). In *C. dubliniensis*, under the same conditions, significant amounts of true hyphae were only observed at 3 h, when NRG1 levels dropped below 40% of those in the YEPD preculture.

*Deletion of CdNRG1 enhances virulence of C. dubliniensis in a macrophage co-culture model*

The growth and survival of the *nrg1Δ/nrg1Δ* mutant CDM10 in co-culture with macrophages was compared to the wild-type strain. In order to have an equal inoculum of yeast cells, both strains were subjected to gentle sonication in a water bath for 5 min prior to inoculation. This treatment broke up any unseparated cells in the *nrg1Δ* mutant. During co-culture with RAW264.7 macrophages, CDM10 was phagocytosed as efficiently as the wild-type *C. dubliniensis* (Fig. 6A). However, while the wild-type *C. dubliniensis* strain failed to produce filaments following phagocytosis, CDM10 produced abundant true hyphae and some pseudohyphae. When the proliferation of CDM10 was compared with the wild-type in the XTT proliferation assay, CDM10 was found to have significantly increased survival and growth compared to the wild type (Fig 6B). Wild-type levels of survival could be restored in CDM10 by reintroduction of the *NRG1* gene from *C. dubliniensis* (strain CDM11) or from *C. albicans* (strain CDM12). These complemented strains formed
some pseudohyphae during co-culture with macrophages, but exhibited greatly reduced growth compared to CDM10 (Fig 6B). The increased proliferation of CDM10 was found to be significantly greater than the \textit{C. dubliniensis} wild-type and reintegrant strains at MOIs of 1:8 and 1:32 (p <0.01). We also examined the virulence of the \textit{C. albicans} \textit{nrg1\textDelta} mutant, MMC3, in this model (Murad \textit{et al.}, 2001b). However, this mutant did not exhibit a phenotype that was significantly different from the parental \textit{C. albicans} strain, as both produced hyphae under the conditions of co-culture and proliferated to a similar extent when measured with the XTT assay (data not shown).

\textit{Deletion of CdNRG1 enhances virulence of \textit{C. dubliniensis} during infection of RHE}

Stokes \textit{et al.} (2007) showed that \textit{C. dubliniensis} is significantly less virulent than \textit{C. albicans} following infection of reconstituted human oral epithelium (RHE) (Schaller \textit{et al.}, 1998). In this study, it was confirmed that \textit{C. dubliniensis} isolates failed to filament during the infection, adhered poorly to the epithelial surface and caused less damage to the epithelial tissue. We compared the virulence of Wü284 with the \textit{CdNRG1}-deletion mutant CDM10 in order to determine if the increased rate of filamentation observed in this strain could enhance RHE tissue damage. The morphology of infecting strains was examined in tissue sections and tissue damage was estimated by measuring the levels of the human enzyme, lactate dehydrogenase (LDH), released from infected epithelial cells. Wü284, typical of wild-type \textit{C. dubliniensis} isolates, grew exclusively in the yeast phase following inoculation onto RHE (Fig. 7A) (Stokes \textit{et al.}, 2007). At 24 h post inoculation, levels of LDH released from tissues infected with strain Wü284 were similar to uninfected controls (Fig. 7B). However, at this time point CDM10 had produced filaments (predominantly
pseudohyphae) and had induced greater levels of LDH release from the infected tissues (Fig. 7B). At 48 h, the integrity of the epithelial surface infected by CDM10 exhibited greater disruption, whereas those infected with wild-type and reintegrated strains were still intact (Fig 7A). The levels of LDH released into the culture medium at this timepoint were also significantly greater in tissues infected with the *nrg1Δ* mutant CDM10 compared with those infected with Wü284 or the reintegrated strain CDM11 (*p* < 0.001, Fig. 7A). The morphology of the reintegrant strain CDM11 was similar to that of the wild-type and induced similar levels of LDH release from infected tissues (Fig. 7B).

*Virulence of a C. dubliniensis nrg1Δ mutant in a systemic mouse infection model*

We compared virulence in the systemic mouse model of infection of *C. dubliniensis* Wü284, the *CdNRG1* deleted strain CDM10 and a derivative with a reintegrated copy of the gene (CDM11). The virulence of all strains was significantly less than that of *C. albicans* SC5314. Similar fungal burdens were recovered in all *C. dubliniensis* strains from the brain, spleen and kidney at two days post-infection (data not shown). Following 28 days infection, no significant difference in survival was observed between the *C. dubliniensis* wild type and *nrg1Δ* mutant strains (mean survival times of 17.8±8.8 and 19±7.1 days) (Fig 8A). Mice infected with *C. albicans* SC5314 in parallel experiments died within 2 days of infection. Candidal burdens in the brain and kidney were assessed for each *C. dubliniensis* strain and no significant difference in load was observed. Due to low burdens in kidneys post infection, wild-type *C. dubliniensis* cells were difficult to find in tissue sections. However, cells that were observed were in the yeast phase, and surrounded by infiltrates of inflammatory cells (Fig. 8B). In contrast, the *nrg1* null strain cells were only found as a single large
clump in a blood vessel of the kidney, consisting of filaments and yeast cells (Fig. 8C).

Discussion

*Candida albicans* has adapted to survive as a commensal organism on human mucosal surfaces (Odds, 1994). However, *C. albicans* is also an opportunistic pathogen, as it can invade and proliferate in many internal organs when the host’s defences are significantly compromised. Its closest relative, *C. dubliniensis* is less frequently recovered from the mucosal surfaces favoured by *C. albicans* and causes fewer opportunistic systemic infections (Sullivan *et al.*, 2004). Identifying the molecular basis for these differences in virulence in two such closely related organisms will provide novel insights into candidal pathogenicity. In this study, we examine the role of the transcriptional repressor Nrg1p in regulating filamentation and virulence in *C. dubliniensis*. *NRG1* was selected for analysis in the present study on the basis of two lines of evidence. Firstly, our own data suggested that positive stimulation of the Ras1p-cAMP pathway with a hyperactive *RAS1* allele or cAMP did not induce filamentation in *C. dubliniensis*. Secondly, in an elegant study, Staib & Morschhäuser (2005) demonstrated that *NRG1* was differentially regulated in *C. dubliniensis* and that this was responsible for pseudohyphal growth and chlamydosporre formation by *C. dubliniensis* on Staib medium. Data presented in the present study suggests that differential regulation of *NRG1* in response to stimuli that promote the growth of hyphae could account in part for the differences in virulence in these two species.
Regulation of CdNRG1

Braun et al. (Braun et al., 2001) first demonstrated that growth of C. albicans in serum at 37°C resulted in downregulation of NRG1 transcript levels. Lotz et al. (Lotz et al., 2004) have also demonstrated pH regulation of NRG1 transcription. In this study, we demonstrated a 90% reduction in NRG1 transcript levels in C. albicans following 1 h in 10% serum at 37°C (Fig. 6). This corresponds well with the levels of germ-tube formation observed under these conditions (~85%, Fig. 4). This downregulation was more rapid than that observed by Braun et al. (2001), possibly due to culture medium differences. In contrast, C. dubliniensis, which filaments at a slower rate in response to serum, downregulates CdNRG1 less rapidly. Under the same conditions, only ~10% of C. dubliniensis cells formed germ-tubes and CdNRG1 transcript levels only dropped by 50% of preculture levels. In these experiments, it took approximately 5 h before transcript levels in C. dubliniensis dropped to below 20% of preculture levels and > 80% of cells had formed germ-tubes. Similarly, following phagocytosis by macrophages, C. albicans rapidly downregulated NRG1 expression whereas in C. dubliniensis, CdNRG1 expression remained constitutively high. These data highlight a fundamental biological difference between C. albicans and C. dubliniensis, namely the ability of C. albicans to rapidly change its morphology via NRG1 downregulation in response to environmental signals, specifically those encountered within the host (e.g. phagocytosis and serum at 37°C). C. albicans has long been described as a fungus that can adapt to many different environments and host niches, and this rapid modulation of NRG1 expression in response to environmental cues may partly explain why C. albicans is more successful and widespread in the human oral cavity than its relative C. dubliniensis.

The rate of filamentation in C. dubliniensis was greatly increased in the nrg1Δ
homozygous deletion strain CDM10, indicating that the slow rate of hypha formation in *C. dubliniensis* is due to *CdNRG1* mediated repression. Deletion of *CdNRG1* also enhanced the rate of filamentation on Spider medium, and in the presence of CO₂, indicating that *CdNRG1* mediated repression is responsible for the lack of filamentation of *C. dubliniensis* under a wide range of environmental conditions tested. Reintroduction of a single copy of *CaNRG1* or *CdNRG1* was sufficient to reverse these phenotypes. Both genes were introduced under the control of their native promoters, indicating that trans-activating factors in the *C. dubliniensis* host strain were responsible for activation of these genes and suppression of the filamentous phenotypes. On Staib medium, both complemented strains formed rough colonies with filamentous fringes, indicating that in this background, the *CaNRG1* and *CdNRG1* genes were regulated similarly.

**Role of CdNRG1 in in vitro models of infection**

We have hypothesised previously that a defect in filamentation may be responsible for the reduced virulence of *C. dubliniensis*. The generation of a homozygous nrg1Δ strain allowed us to test this hypothesis. We initially examined the virulence of *C. dubliniensis* in co-culture with RAW264.7 macrophages (Marcil et al., 2002). Wild-type *C. dubliniensis* was significantly less able to proliferate in co-culture with these cells in comparison to *C. albicans*, and this was associated with low levels of filamentation following phagocytosis and constitutive expression of *CdNRG1* (Fig. 7). Deletion of *CdNRG1* greatly increased proliferation and filamentation of *C. dubliniensis* in this model. The reason for this increased proliferation may be two-fold. Increased filamentation allows *C. dubliniensis* cells to escape from the phagolysosome in a fashion similar to *C. albicans* cells (Lo et al., 1997; Marcil et al.,
2002). A second possibility is that deletion of CdNRG1 may alter the expression of many stress-response genes. Indeed, Murad et al. (2001) demonstrated that the *C. albicans nrg1Δ* strain had increased sensitivity to H$_2$O$_2$. However, the CdNRG1 mutant did not display increased sensitivity to H$_2$O$_2$ or nitric oxide *in vitro* in our assays, indicating possible differences in the sets of genes regulated in both species.

In a second *in vitro* model, using reconstituted human oral epithelium, the *C. dubliniensis CdNRG1* mutant also displayed increased virulence relative to the wild-type strain. This system has previously been shown to be a sensitive model of virulence, in which filamentation and proteinase secretion play important roles in tissue penetration (Schaller et al., 1999). Following 24 h growth on the tissues, the wild-type *C. dubliniensis* strain had failed to filament and adhered poorly to the tissue, as previously shown by Stokes *et al.* (2007). In addition, the levels of LDH release (a marker for epithelial cell disruption) induced by this wild-type strain were similar to uninfected control tissues. Following 48 h incubation, the CdNRG1 mutant had formed filaments, and had caused significantly more damage than wild-type *C. dubliniensis*. The role of filamentation in *C. albicans* during oral colonisation and infection has been well established, and this property is associated with strong adherence and tissue penetration (Bernhardt et al., 2001). The lack of adherence and penetration exhibited by the wild-type *C. dubliniensis* strain on RHE offers an explanation for the low incidence of colonisation and infection caused by *C. dubliniensis* on the oral niches favoured by *C. albicans* (i.e. the dorsum of the tongue, the oesophagus and the palate). Secondly, the increased susceptibility of *C. dubliniensis* to killing by macrophages may also prevent this organism invading and penetrating oral tissues to cause infection. Macrophages are an important part of the cell-mediated immune response in the oral cavity, and this phenotype may explain
why wild-type *C. dubliniensis* mainly causes infection in patients with severe defects in cell-mediated immunity (Challacombe and Sweet, 2002; Sullivan *et al.*, 2004).

**Role of CdNRG1 during systemic infection**

The standard model of systemic virulence in *Candida* spp. involves intravenous inoculation of the organism into mice (MacCallum and Odds, 2005). It has been established that *C. dubliniensis* requires a higher inoculum than *C. albicans* to establish infection in this model (1x10⁵ cfu/g body weight per mouse; D. MacCallum, unpublished data). This level of inoculum led to the death of all *C. albicans*-infected mice within two days, whereas one third of *C. dubliniensis*-infected mice were still alive by day 28. Infection with the *C. dubliniensis* CdNRG1 mutant did not lead to any significant change in survival or organ burdens compared to wild-type. This is a complex model of infection compared to the *in vitro* models used previously in this study. In order to establish infection in this model, the organism must evade neutrophil killing, penetrate the endothelium and escape from the bloodstream and then penetrate and invade target organs such as the kidney and the brain (MacCallum and Odds, 2005). The low virulence of wild-type *C. dubliniensis* in this model suggests that there may be a fundamental virulence defect in one of these processes that cannot be overcome by simply increasing the rate of filamentation. Recent comparative genomic studies have highlighted other possible reasons for the reduced virulence of *C. dubliniensis* in this model (Moran *et al.*, 2004). A significant number of *C. albicans* genes present on Eurogentec DNA microarrays (~4%) appear to have either no orthologue in *C. dubliniensis*, or one with significant divergence at the nucleotide sequence level (Moran *et al.*, 2004). In particular, these studies have shown that *C. dubliniensis* possesses only one orthologous gene of the SAP4,5,6 subfamily of
secreted aspartyl proteinases, namely \textit{CdSAP4}. These proteinases are expressed in the hyphae of \textit{C. albicans}, and deletion of \textit{SAP6} reduced invasion of parenchymal organs and deletion of all three genes renders \textit{C. albicans} attenuated in the systemic model of infection (Felk \textit{et al.}, 2002; Sanglard \textit{et al.}, 1997). We are currently carrying out heterologous expression studies in \textit{C. dubliniensis} in order to determine the significance of this defect.

\textit{Conclusion}

\textit{C. dubliniensis} is the closest known relative of \textit{C. albicans}, yet is clinically less significant and has reduced virulence in models of infection. We have demonstrated that the greater virulence of \textit{C. albicans} relative to \textit{C. dubliniensis} is partly due to its ability to rapidly downregulate \textit{NRG1} in response to environmental signals encountered in vivo. Expression of the \textit{CdNRG1} repressor is responsible for the lack of filamentation of \textit{C. dubliniensis} under many conditions, and that deletion of this repressor can increase filamentation and virulence in co-culture with macrophages and in a model of epithelial tissue invasion. The increased morphological plasticity of \textit{C. albicans} may allow this species to colonise a wider range of mucosal sites. In addition, this may indicate that \textit{C. dubliniensis} has specialised to inhabit a unique niche where filamentation is not required, or may perhaps even be disadvantageous. Along with comparative molecular and genomic approaches, discovery of the preferred niche of \textit{C. dubliniensis} should go a long way to explaining the reason for the phenotypic differences between these two species.
Experimental Procedures

Candida Strains and growth conditions

All Candida strains were routinely cultured on yeast extract-peptone-dextrose (YEPD) agar, at 37°C. For liquid culture, cells were grown shaking (200 r.p.m.) in YEPD broth, also at 37°C (Gallagher et al., 1992). Genotypes of strains used in this study are listed in Table 1. Several solid media were used to induce filamentation. Spider medium and synthetic low dextrose (SLD) medium were prepared as described (Lui et al., 1994). Tobacco medium was prepared as described (Khan et al., 2004). Induction of filamentation in liquid cultures was carried out with cells from overnight YEPD broth cultures (37°C), which were washed twice with sterile Milli-Q water and added to prewarmed hyphal-induction medium to a density of 2 x 10^6 cells/ml. Induction media used here include Dulbecco’s modified Eagle’s medium (DMEM, Sigma-Aldrich Ireland Ltd) and sterile Milli-Q (Millipore Ireland B.V., Co. Cork, Ireland) water supplemented with 10% (v/v) or 50% (v/v) foetal bovine serum (Sigma-Aldrich). The proportion of unconstricted germ-tubes in each culture was assessed at 30 min intervals by microscopic examination with a Nikon Eclipse 600 microscope (Nikon U.K., Surrey, U.K.).

Macrophage cell culture and infection with Candida

The murine macrophage-like cell line RAW264.7 was obtained from the American Type Tissue Culture Collection (ATCC). The complete medium used to maintain the cell line consisted of DMEM supplemented with 10% (v/v) heat-inactivated foetal bovine serum (Sigma-Aldrich) and 100 U/ml penicillin and 100 mg/ml streptomycin (Sigma-Aldrich). Cells were grown at 37°C under an atmosphere with 5% (v/v) CO₂ in untreated 90 mm diameter plastic dishes (Sarstedt, Co. Wexford, Ireland).
Co-culture experiments with *Candida* and RAW264.7 macrophages were carried out in flat-bottomed 96-well dishes (Sarstedt). For each experiment, a macrophage suspension of $1.5 \times 10^6$ cells/ml was prepared in complete medium. For each *Candida* strain to be tested, 16 wells (4 x 4) were dispensed with 100 µl of this suspension and cultured overnight (16 h) to form confluent monolayers. Prior to experiments, confluent monolayers were washed with fresh complete medium and a final volume of 150 µl medium was added to each well. *Candida* strains were cultured overnight in 2 ml YEPD broth in a 25 ml tube at 37˚C with shaking. Cells were washed twice in Dulbecco’s modified phosphate buffered saline (DPBS, Sigma-Aldrich) and a suspension of $1.5 \times 10^6$ cells/ml was prepared in complete medium. A 50 µl aliquot of this suspension was added to quadruplicate wells containing confluent washed monolayers and quadruplicate wells containing 150 µl of complete medium alone. This yielded $7.5 \times 10^4$ *Candida* cells per well, giving a multiplicity of infection (MOI) of 1:2 (*Candida*:macrophages). Cells from this well (50 µl) were then serially diluted 1:4 in the three adjacent wells to yield MOIs of 1:8, 1:32 and 1:128, respectively. Wells containing complete medium alone acted as macrophage-free growth controls in all experiments. The inoculated plates were incubated at 37˚C in 5% (v/v) CO$_2$ for 16 h. Following incubation, the survival of the *Candida* cells was assessed by comparing their growth to the macrophage free control wells using an XTT (Sigma-Aldrich) dye reduction assay. Briefly, each well was washed three times with sterile water which resulted in lysis of the macrophages in those wells containing monolayers. Cells were then incubated with 200 µl of a 400 µg/ml XTT solution containing co-enzyme Q (50 µg/ml) for 45 min. Following incubation, 100 µl of the reduced dye solution was removed and the absorbance measured at 480 nm in a Tecan Genios microplate reader (Tecan U.K. Ltd., Reading, U.K.). Absorbance values were
corrected against a cell-free blank and the percentage growth of each *Candida* strains was determined relative to the positive growth control (100%) for that MOI. Each experiment was performed on at least four occasions. Proliferation curves were analysed with 2-way ANOVA to determine if strains exhibited significant differences in proliferation at each MOI, using the Prism 4.0 software package (GraphPad Software, San Diego, CA). Staining of *Candida*-infected macrophages with acridine orange was carried out with infected macrophage monolayers grown on sterile glass coverslips. The medium was removed and a solution of acridine orange (10 μg/ml) in DPBS was added to duplicate wells and incubated for 10 min. The stain was then removed and the cells washed once in DPBS. Fluorescence was detected using a Nikon Eclipse 600 microscope (Nikon) fitted with a super high power mercury lamp (Nikon) and the GFP specific filter set (Endow GFP Bandpass Emission (FGP®-BP) filter combination), (Nikon).

**Infection of Reconstituted human oral epithelial (RHE) tissues**

RHE tissues were purchased from Skinethic Laboratories (Nice, France) and used as described previously (Schaller *et al.*, 1999; Stokes *et al.*, 2007). Reconstituted epithelium samples (0.5 cm²) were inoculated with 2 x 10⁶ yeast cells in 50 μl of PBS and controls were inoculated with 50 μl of PBS. Inoculated cultures were incubated at 37°C, 5% (v/v) CO₂ at 100% humidity for 6 h, 12 h, 24h and 48 h. The release of lactate dehydrogenase (LDH) from epithelial cells into the cell-culture medium was measured to quantify the extent of epithelial cell damage. The CytoTox 96® non-radioactive cytotoxicity assay (Promega Corp., Madison, WI) was used to measure the amount of LDH in each sample. The reaction was assayed at 480 nm using a Genios plate reader (Tecan U.K. Ltd.). One unit of LDH activity is equivalent to 1 μM
formazan formed per reaction. The statistical significance of differences in LDH release induced by each strain at each time-point was examined by 2-way ANOVA. Sectioning and staining of tissues for light microscopy was carried out as described by Stokes et al. (2007). Tissues were examined using a Nikon Eclipse 600 microscope. Infections were carried out on two separate occasions.

_Candida transformation_

Transformation of _C. albicans_ and _C. dubliniensis_ was performed by electroporation as described (Moran et al., 2002). The _URA3_ deleted strains CAI4 and CDUM4B were transformed with the _Candida ARS_-containing plasmids pVEC and pLJ65 and transformants were selected on selective minimal medium containing 1% (w/v) casamino acids, as described (Leberer et al., 2001). Deletion of the _C. dubliniensis NRG1_ gene in strain Wü284 was carried out using the _SAT1_-flipper described by (Reuss et al., 2004). A deletion construct was created by PCR by amplifying the flanking 5' and 3' regions of the _CdNRG1_ gene with the primer pairs NRGKF/NRGXR and NRGSCIIF/NRGSIR, respectively (Table 2). These 5' and 3' fragments were cloned into the _KpnI/XhoI_ and _SacII/SacI_ sites, respectively, flanking the _SAT1_-flipper in plasmid pSFS2A to yield pGM142. The entire deletion construct was released from plasmid pGM142 on a _KpnI-SacII_ fragment and used to transform _C. dubliniensis_ Wü284 as described previously (Moran et al., 2002). Nourseothricin-resistant recombinant derivatives were selected on YEPD agar plates containing 100 µg/ml nourseothricin. Integration of the construct at the correct locus was initially confirmed by PCR with the primer pair NRGUP (which bound to upstream chromosomal sequences, Table 2) and FLP1 (which bound to _SAT1_ cassette sequences), which yield a product of approximately 1,400 bp in transformants
containing the cassette at the CdNRG1 locus. The marker was excised and recycled for a second round of transformation as described by Reuss et al. (2004). This resulted in the generation of strain CDM10, which contained a deletion between nucleotides -74 and +949 (where the first A of the ATG start codon is +1) in both copies of the CdNRG1 gene. This was confirmed by Southern blot analysis (Figure 3). Reintroduction of the wild-type CdNRG1 gene was achieved by PCR amplification of regions –3849 to +1423 of the NRG1 gene, which was ligated into NolI and SacII sites of the C. dubliiniensis integrating vector pCDRI to yield pCdNRG1. Plasmid pCDRI consists of plasmid pBluescript II containing the SAT1 resistance marker from pSFS2A (contained on an XbaI/SpeI fragment) and regions +1800 to +2488 of the CdCDR1 pseudogene. Targeted integration of pNRG1 into the CdCDR1 pseudogene in strain CDM10 was achieved by linearisation of the plasmids within the CdCDR1 region by digestion with NcoI and transformation into CDM10 by electroporation, yielding the CdNRG1-complemented strain CDM11. The correct genomic integration was confirmed by PCR, using a primer that annealed within the cassette (M13 reverse primer) and a primer that annealed within the CdCDR1 sequences (TAGR) (Moran et al., 2002). The presence of the whole CdNRG1 gene within the integrated cassette was also confirmed by PCR with the NRGKF and NRGSCIR primer pair. The homozygous nrg1Δ strain CDM10 was also transformed with the empty vector pCDRI for use in parallel experiments with the reintegrant CDM11.

Real-time PCR analysis of gene expression

Cells were harvested from Candida-macrohage co-culture experiments or liquid hypha induction medium and frozen directly in liquid nitrogen for RNA preparation. RNA was prepared using TRI-reagent (Sigma) as described (Stokes et al., 2007).
RNA samples were rendered DNA free by incubation with Turbo-RNAfree reagent (Ambion, Austin, TX). RNA concentrations were determined using the Ribo-green kit (Molecular Probes). Reverse transcription was carried out with 1 µg of total RNA with an oligo-dT primer (Promega) and Superscript II reverse transcriptase (Invitrogen, Carlsbad, CA). Amplification was carried out using the primers pairs QRTEF1F/R (Green et al., 2005) and the primers NRG1A/B (Table 2). NRG1A/B primers were designed using Primer Express software v1.5 (Applied Biosystems, Foster City, CA) and were homologous to C. albicans and C. dubliniensis NRG1 ORFs. These primers yielded single, specific amplimers from genomic DNA and cDNA templates from both C. albicans and C. dubliniensis. Real-time detection of TEF1 and NRG1 amplimers was carried out using the QuantiTect Sybr green PCR kit (Qiagen, West Sussex, U.K.) and the ABI 7700 sequence detector, performing separate reactions for each gene. NRG1 gene expression levels were normalised against the expression levels of the constitutively expressed TEF1 gene in the same cDNA sample.

Construction of GFP reporter strains

C. albicans and C. dubliniensis strains were constructed with one allele of the NRG1-coding sequence replaced by the yeast-optimised GFP (yGFP3) coding region using cassettes containing yGFP3 and the SAT1 marker gene (Cormack et al., 1997; Reuss et al., 2004). In order to create these constructs, the yGFP3 coding region was amplified from plasmid pCC1 (Stokes et al., 2007) using primers GFPHF and GFPHR (Table 2), digested with HindIII and EcoRV and ligated into HindIII/EcoRV-digested pBluescript II. The ACT1 terminator region was released from pCC1 by EcoRI digestion and this fragment was subcloned in the EcoRI site downstream of the
GFP ORF to create pGM160. The SAT1-resistance marker was released from pSFS2A by digestion with SpeI and XbaI and this fragment ligated to SpeI/XbaI-digested pGM160 to create pGM161. In order to create CaNRG1 and CdNRG1 GFP-promoter fusion cassettes, upstream 5′ regions of the CaNRG1 and CdNRG1 genes were amplified using the primer pairs PCaNRGF/R and PCdNRGF/R, respectively (Table 2). These primers were similar to primers pairs NRG1P1/P2 and CdNRG1P1/P2 described by Staib & Morschhäuser (2005), except that the internal restriction endonuclease recognition sequences were changed. These fragments were digested with KpnI and XhoI and inserted into KpnI/XhoI-digested pGM161 to create separate C. albicans and C. dubliniensis NRG1 promoter-GFP fusions. The 3′ non-coding regions of the CaNRG1 and CdNRG1 genes were then amplified with the primer pairs CaNRG3F/R and CdNRG3F/R respectively (Table 2) and these products were ligated into the corresponding SacII/SacI-digested CaNRG1 or CdNRG1 construct to create plasmids pCaNRG1GFP and pCdNRG1GFP. The cassettes were released by digestion with KpnI and SacI and were used to replace one copy of the NRG1 gene by targeted gene replacement in C. dubliniensis Wü284 and C. albicans SC5314. PCR confirmation of the correct allelic replacement was obtained using a primer binding to the NRG1 promoter (NRGKF, Table 2) and a primer binding within the cassette (ACTTR, Stokes et al., 2007)

Mouse model of systemic candidosis

For virulence testing of strains, immunocompetent 6–8 week old female BALB/c mice (Harlan Sera-Lab Ltd., Loughborough, UK) were challenged intravenously. Fungal strains were grown with shaking for 18–24 h in NGY medium (0.1% [w/v] neopeptone, 0.4% [w/v] glucose, and 0.1% [w/v] yeast extract) at 30°C. Cells were
sonicated prior to inoculum preparation to disrupt clumps, washed twice with sterile water and resuspended in physiological saline. Groups of eight mice were inoculated intravenously with each strain at $1 \times 10^5$ cfu/g of mouse body weight. Two mice were sampled at two days post-infection to determine organ burdens at this time point. The remaining 6 mice per group were monitored over 28 days when the experiment was terminated. Any mice showing signs of severe illness were terminated prior to the end of the experiment. For each mouse, the kidneys and brains were aseptically removed post mortem, divided in half, and one half homogenized in 0.5 ml of water, and Candida tissue burdens determined by viable counting. Survival curves were compared using the logrank test (Prism 4.0, GraphPad software). For histology, the other half of each organ was preserved in formalin. Tissues were paraffin-embedded and 5 µm sections cut. Sections were deparaffinised and stained with Periodic Acid-Schiff, prior to staining with haematoxylin.

**Acknowledgements**

We thank Jan Walker, Dept. of Histopathology, St James’s Hospital Dublin for histological preparation of RHE tissues. This work was supported by the Irish Health Research Board (Grant no. RP/2004/235) and Science Foundation Ireland (Grant no. 04/IN3/B463). We would like to thank Alistair Brown for the gift of *C. albicans* strain MMC3 and Malcolm Whiteway for the gift of plasmids pVEC and pLJ65.
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Figure Legends

Fig. 1. Phenotypes of *C. albicans* (A) and *C. dubliniensis* (B) following 3 h co-culture with murine macrophage cell line RAW264.7. Macrophage cells were grown on cover-slips and infected with *Candida* spp. at an MOI of 1:2 for 3 h, fixed in methanol and stained with Giemsa. The black scale bar represents 10 µm (C) Proliferation of *C. albicans* isolates (n=6) and *C. dubliniensis* genotype I isolates (n=5) and genotype II isolates (n=4) in co-culture with RAW264.6 macrophages. Proliferation of *Candida* isolates alone (control) and in co-culture was estimated following 16 h incubation using an XTT dye reduction assay. Percentage growth of co-cultured *Candida* was estimated relative to the macrophage-free control cultures. MOIs where *C. albicans* isolates yielded significantly greater levels of proliferation are indicated indicated by ‘**’ (P <0.001).

Fig. 2. Photomicrographs of *C. albicans* CAI4 and *C. dubliniensis* CDUM4B cells transformed with pVEC and pLJ65 (A) and grown in the presence of cAMP (B). (A) *C. albicans* CAI4 and *C. dubliniensis* CDUM4B were transformed with the *CaARS* plasmid pVEC and a derivative harbouring a hyperactive *RAS1* allele (pLJ65). Cells were grown in 1% (w/v) casamino acids to induce expression of *RAS1* from the *PCK1* promoter. (B) *C. albicans* SC5314 and *C. dubliniensis* Wü284 were grown in DMEM with and without 10 mM dibutyryl-cAMP.

Fig. 3. Creation of a homozygous deletion in *CdNRG1* in strain Wü284. Sequential disruptions in both alleles were created with the SAT1-flipper. Genomic DNA from transformants was isolated as described (Gallagher et al., 1992) and digested with *EcoRI* and fragments were resolved on a 1% (w/v) agarose gel. Southern-blotted
DNA was hybridised with a fragment corresponding to the 5' end of the CdNRG1 gene, amplified with primers NRGUP and NRGXR and labelled by random priming with $[^{32}\text{P}]$dATP (6,000 Ci/mmol; 222 TBq/mmol, Amersham International Plc).

**Fig. 4.** Phenotypic analysis of the CdNRG1 homozygous deletant strain CDM10. (A) Colonial morphologies of *C. albicans* SC5314, *C. dubliniensis* Wü284 and CDM10 (*nrg1Δ/nrg1Δ*) on solid media. (B) Photomicrographs of *C. albicans* SC5314, *C. dubliniensis* Wü284 and CDM10 (*nrg1Δ/nrg1Δ*) grown in liquid DMEM in the presence of 5% (v/v) CO$_2$. (C) Rate of true hypha formation in *C. albicans* SC5314, *C. dubliniensis* Wü284 and CDM10 (*nrg1Δ/nrg1Δ*) in water containing 50% (v/v) foetal bovine serum.

**Fig. 5.** (A) Analysis of NRG1 promoter activity using NRG1 promoter-GFP fusion strains of *C. albicans* SC5314 and *C. dubliniensis* Wü284. Strains were co-cultured with RAW264.7 macrophages and fluorescence was examined over time with a Nikon Eclipse 600 microscope (Nikon) fitted with a GFP specific filter set (Endow GFP Bandpass Emission (FGP®-BP) filter combination), (Nikon). (B) Analysis of NRG1 transcript levels in *C. albicans* SC5314 and *C. dubliniensis* Wü284 during co-culture with Raw264.7 macrophages by real-time PCR, using the Quantitect SYBR Green PCR system (Qiagen) (C) Analysis of NRG1 transcript levels in *C. albicans* SC5314 and *C. dubliniensis* Wü284 during growth in 10% foetal calf serum.

**Fig. 6.** Analysis of the effects of deletion of CdNRG1 during co-culture with macrophages. (A) Photomicrographs of *C. albicans* SC5314, *C. dubliniensis* Wü284 and CDM10 (*nrg1Δ/nrg1Δ*) during co-culture with murine macrophages over 5 h
following staining with acridine orange. The white scale bar represents 10 µm (B) Proliferation of *C. dubliniensis* Wü284, CDM10 (*nrg1Δ/nrg1Δ*) and the complemented mutant strain CDM11 in the presence of RAW264.7 macrophages. Proliferation was estimated in an XTT dye reduction assay. Points marked ‘*’ indicate MOIs where the mutant strain proliferated to a significantly greater extent than wild-type and reintegrant strains (P <0.01).

**Fig. 7.** (A) Photomicrograph of *C. albicans* SC5314, *C. dubliniensis* Wü284, CDM10 (*nrg1Δ/nrg1Δ*) and CDM11 (*nrg1Δ/nrg1Δ*, pNRG1) after 24 h growth on reconstituted human oral epithelium (RHE; Skinetic, France). Histological sections were stained with Periodic-Acid Schiff reagent. The black scale bar represents 40 µm (B) Evaluation of tissue damage caused during infection of RHE by *C. albicans* SC5314, *C. dubliniensis* Wü284 and CDM10 (*nrg1Δ/nrg1Δ*) and CDM11 (*nrg1Δ/nrg1Δ*, pNRG1) measured as LDH activity release into culture medium. Tissues infected with the *nrg1Δ* mutant strain CDM10 released significantly higher levels of LDH relative to wild-type and reintegrant strains at 48 h (P< 0.001, indicated as *).

**Fig. 8.** (A) Survival curves for mice infected intravenously with *C. albicans* SC5314, *C. dubliniensis* Wü284, CDM10 (*nrg1Δ/nrg1Δ*) and CDM11 (*nrg1Δ/nrg1Δ*, pNRG1). The experiment was terminated at day 28. (B and C) Histological sections of kidneys stained with periodic-acid Schiff, and post-stained with haematoxylin after infection with *C. dubliniensis* Wü284 (B) and CDM10 (*nrg1Δ/nrg1Δ*) (C). Yeast cells in (B) are marked with arrowheads. Scale bar represents 20 µm.
**Fig. 1**

A  

B  

C. albicans (n=6)  
C. dubliniensis genotype I (n=5)  
C. dubliniensis genotype II (n=4)

MOI (Candida:Macrophage)

% growth relative to control

![Graph](image)

- **C. albicans (n=6)**
- **C. dubliniensis genotype I (n=5)**
- **C. dubliniensis genotype II (n=4)**
Fig. 2

A

CAI4

CDUM4B

B

SC5314

Wü284
Fig. 3.

Kb

1 2 3 4 5

NRG1
nrg1Δ::FRT
nrg1Δ::SAT1-FLIP

10
5
3
Fig. 4

A  

YPD, 37˚C

YNB + FCS, 37˚C

Spider, 30˚C

B

DMEM + cAMP

DMEM + CO₂

SC5314
Wu284
CDM10 (nrg1Δ)

% hyphal formation

Time (h)
**Fig. 5**

A  

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<tr>
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<th>phase contrast</th>
<th>fluorescence</th>
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<td><img src="image2" alt="fluorescence" /></td>
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<tr>
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<td>Wü284, 5 h</td>
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B  

![Graph B](image9)

C  

![Graph C](image10)
Fig. 6

A

SC5314  Wü284  CDM10 (nrg1Δ)

0.5 h

3 h

5 h

B

% growth relative control

MOI (Candida:Macrophages)

MOI (Candida:Macrophages)

Wü284

CDM10 (nrg1Δ/nrg1Δ)

CDM11 (CDM10 + pCdNRG1)

CDM12 (CDM10 + pCaNRG1)

0 25 50 75 100

*
Fig. 7

A

Wü285  CDM10  CDM11

nrg1Δ/nrg1Δ  nrg1Δ/nrg1Δ  nrg1Δ+pCdNRG1

B

LDH U

Hours post infection

Uninfected  WÜ284  CDM10 (nrg1Δ, pCDRI)  CDM11 (nrg1Δ, pNRG1)
Fig. 8.

A

B

C

Days elapsed
0 5 10 15 20 25 30

Percent survival
0 25 50 75 100

SC5314
WÜ284
CDM10 (nrg1Δ, pCDRI)
CDM11 (nrg1Δ, pNRG1)