The Candida albicans-Specific Gene EED1 Encodes a Key Regulator of Hyphal Extension

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Abstract

The extension of germ tubes into elongated hyphae by Candida albicans is essential for damage of host cells. The C. albicans-specific gene EED1 plays a crucial role in this extension and maintenance of filamentous growth. eed1Δ cells failed to extend germ tubes into long filaments and switched back to yeast growth after 3 h of incubation during growth on plastic surfaces. Expression of EED1 is regulated by the transcription factor Efg1 and ectopic overexpression of EED1 restored filamentation in efg1Δ. Transcriptional profiling of eed1Δ during infection of oral tissue revealed down-regulation of hyphal associated genes including UME6, encoding another key transcriptional factor. Ectopic overexpression of EED1 or UME6 rescued filamentation and damage potential in eed1Δ. Transcriptional profiling during overexpression of UME6 identified subsets of genes regulated by Eed1 or Ume6. These data suggest that Eed1 and Ume6 act in a pathway regulating maintenance of hyphal growth thereby repressing hyphal-to-yeast transition and permitting dissemination of C. albicans within epithelial tissues.

Introduction

Candida albicans is normally a harmless commensal and part of the microflora on mucosal surfaces, but frequently causes superficial infections such as oral or vaginal thrush. Infection of epithelial surfaces is associated with extensive growth, invasion into and dissemination within epithelial tissues and inflammation. In some circumstances, for example after organ transplantation, the fungus may cause invasive and life threatening systemic infections. In these patients, the fungus can disseminate, usually from the gastrointestinal tract or from biofilms on medical devices, via the bloodstream to deeper cell layers and disseminate, as shown previously for oral epithelial tissue [12].

Several virulence attributes of C. albicans are considered to play roles during invasion of human cells. One of the most important invasive properties of C. albicans is the ability to change growth morphology from spherical yeast cells to elongated hyphae (dimorphism). Hyphae are not only essential for invasion [2], but are also more adhesive to host cells as compared to the yeast growth form [3]. Attachment of C. albicans hyphae to epithelial surfaces is mediated by hyphal-associated invasins, namely the glycosylphosphatidylinositol (GPI)-anchored protein Hwp1 [4] and members of the GPI-anchored agglutinin-like Als protein family of invasion, while enterocytes are only invaded by active penetration [8]. The Als family member Als3 acts as a C. albicans invasion triggering fungal up-take via interactions with receptors on epithelial or endothelial cells [9]. Several other hyphal-associated factors may have specific functions during attachment and invasion of host cells [10,11]. After initial invasion into superficial epithelial cells, fungal hyphae can penetrate into deeper cell layers and disseminate, as shown previously for oral epithelial tissue [12].

Many studies have investigated the environmental conditions, which induce the yeast-to-hyphal transition of C. albicans in vitro. Known inducers of hyphal formation are a temperature shift to 37°C, addition of serum, the increase of environmental pH to physiological values between pH 6 and 7, hypoxic conditions, physiological CO2 concentrations or contact with surfaces ranging from plastic (e.g. catheters) to human host cells [13–15]. Furthermore, ingestion by macrophages can induce hyphal growth of C. albicans as part of an escape mechanism: phagocytosed yeast cells can produce hyphae, which premiere the host membrane and kill the macrophage [16,17]. In addition, the transition between yeast and hyphal growth forms has a significant effect on the host immune response and possibly on the outcome of an infection [18,19]. Therefore, hyphae seem to be not only essential for adhesion and invasion, but also for immune evasion. Consequently, nonfilamentous mutants are strongly reduced in virulence [16]. However, hyperfilamentous mutants which lack the hyphal...
repressors Nrg1 or Tup1 and which cannot grow in the yeast form are also reduced in virulence [20,21]. This suggests that morphological plasticity and the ability to grow either in the yeast or the hyphal phase are essential for virulence of C. albicans. Due to the importance of the yeast-to-hyphal transition for virulence of C. albicans, multiple studies have investigated the molecular and cellular events associated with this morphological transition. Signal transduction pathways controlling hyphal formation such as the mitogen-activated protein (MAP) kinase cascade and the cAMP pathway converge at the transcription factors Eps1 and Efg1 which together are crucial for hyphal formation and the activation of hyphal-associated genes [14,15,22]. Other cellular factors such as the Rho-GTPase Cdc42 and the cyclin Hgc1 play further important roles in the regulation of filamentous growth of C. albicans. Hgc1 has been shown to phosphorylate Efg1 and mutants lacking Hgc1 fail to form true hyphae, but still express certain hyphal-associated genes [23–25]. Although much is known about hyphal induction of C. albicans, regulation of hyphal extension is less well studied and despite intensive research on dimorphism, it is still poorly understood how the switch from hyphal-to-yeast growth is regulated. Only a few genes, such as PES1, which encodes a C. albicans pescadillo homolog, were shown to be involved in the switch from hyphae to yeast cells [26]. Furthermore, the consequences of a dimorphic switch on pathogenesis remain unclear. More recent studies have identified another key transcription factor, Ume6, which is necessary for the extension of germ tubes into hyphae [27]. Overexpression of UME6 can restore filamentation in several mutants which are unable to form hyphal [28].

We recently identified a C. albicans gene of previous unknown function, which was expressed during oral tissue infections and in patients suffering from oral infections. Mutants lacking this gene were able to invade superficial oral epithelial cells, but once inside a host cell, the mutants grew as yeast cells, remained trapped intracellularly and did not disseminate within epithelial tissue. Therefore, the gene was named EED1 (Epithelial Escape and Dissemination 1) [12]. Here, we show that Eed1 is a unique protein of C. albicans and essential for hyphal extension on solid surfaces and during interaction with host cells. Expression of UME6 depends on Eed1, which itself is a target of the transcription factor Efg1, and ectopic overexpression of UME6 restored hyphal elongation in eed1Δ. We suggest that Eed1 and Ume6 act in a pathway which controls the extension of germ tubes into hyphae, the hyphal-to-yeast transition and escape from non-phagocytic host cells.

**Results**

**EED1 is unique to C. albicans**

Using Blast searches within the available genomic sequences we aimed to identify homologues of EED1. The closest hit was DEFI, coding for a regulator of RNA polymerase II (RNAPII) with multiple functions in S. cerevisiae [12,29,30]. In fact, Ecd1 has structural similarities with Def1: both proteins are of comparable length and unusually rich in glutamine residues over a 200–300 amino acid central region [12]. However, we found the overall identity (13%) and similarity (18.2%) were low and no homology was found flanking the glutamine rich region, suggesting that both genes have evolved independently and are likely to have different functions. No homolog of EED1 was detected in the genomes of species within the CUG clade, including very close relatives of C. albicans [31]. This was despite the fact that the gene locus of EED1 in C. albicans (containing genes *ITA6, BET2, DPB2*, and *GPN3*) is conserved within the CUG species, although the gene order differs (not shown). The closest relative of C. albicans, C. dubliniensis, contains a syntenic gene between *ITA6* and *BET2* named *MID1* (Moran et al., unpublished data). However, the overall identity between Eed1 and Mid1 is low (13.4%), with a slightly higher similarity (26.6%) than between Eed1 and Def1. These data suggest that Eed1 is unique to C. albicans.

**Dynamics of transient filamentous growth of eed1Δ on plastic surfaces**

Mutants lacking *EED1* were unable to produce true hyphae in liquid media and only transiently produced filaments during contact with oral epithelial cells, but switched to yeast cell growth during the infection process [12]. Induction of hyphal formation of *eed1Δ* during co-cultivation with epithelial cells was dependent on contact with epithelial cells, but also occurred after contact with other surfaces (e.g. plastic). To study the dynamics of this contact-dependent, transient filamentation of *eed1Δ* cells in more detail, we analyzed growth of wild type and mutant cells on plastic surfaces in RPMI medium via time lapse microscopy.

Since the triple auxotrophic wild type strain BWP17 was used to produce the *eed1Δ* mutant [12] and since differences between the parental C. albicans wild type SC5314 and the derivative BWP17 are sometimes observed, we compared these two strains in all assays used in this study. For comparison, we used an auxotrophic version of BWP17 carrying the plasmid pGIP30 (see Material and Methods). Both strains behaved similarly in all assays and no differences were observed. Therefore, we used strain SC5314 in all further experiments as a wild type control.

In addition to the *eed1Δ* mutant described in Zakikhany et al. [12] we also have produced an *eed1Δ* mutant with SC5314 as a parental strain. Regardless of the strain background, either BWP17 or SC5314, *eed1Δ* mutants showed similar phenotypes throughout this study (data not shown). The growth rate of *eed1Δ* mutant and wild type yeast cells was similar (not shown).

Supplemental movies S1 and S2 and Fig. 1 clearly show that both wild type and mutant cells respond to contact to plastic surfaces in RPMI medium by forming germ tubes. However, after 3 h, wild type germ tubes continued to extend and occasionally showed branching formations, while *eed1Δ* germ tubes did not form such structures. Instead we observed budding of yeast cells from the initial filaments (“budding filaments”) (Supplemental movies S1 and S2, Fig. 1). Over time, the wild type strain maintained hyphal growth and produced a dense mycelium at time point 12 h. In sharp contrast, the entire population of *eed1Δ* cells grew as yeast cells after 12 h (Fig. 1, 12 h). These morphological differences were accompanied by dramatic differential adhesion properties. While germ tubes of wild type and *eed1Δ* cells adhered to the plastic surface, budding filaments almost completely lost their adherence properties and yeast cells were released from the surfaces (Supplemental movies S1 and S2). These altered phenotypes of *eed1Δ* cells indicate not only differences in the morphology, but also in the expression of hyphal associated adhesins.

**Analysis of septation and budding events in primary eed1Δ filaments**

To further analyze the cellular morphology and dynamics of “budding filaments” of *eed1Δ* as compared to wild type cells, we questioned whether primary filaments were able to produce septa. In order to stain septae with Calcofluor White, wild type and mutant cells were grown on glass surfaces with RPMI1640 medium at 37°C. As shown in Figure 2, both wild type and *eed1Δ* cells formed filaments with septa (Fig. 2, 6 h, septae marked by arrows). Budding of *eed1Δ* yeast cells from primary filaments...
occurred predominantly from sites of septation (Fig. 2, 6 h, 8 h, marked by arrows). These results suggest that septum formation is not affected by the deletion of EED1, that true hyphae are formed after contact to surfaces and that budding of yeast cells from filaments occurs after septation.

Identification of genes regulated by EED1

To identify genes whose expression is influenced by the activity of EED1 during interaction with host epithelial cells, we analyzed the genome wide gene expression profiles of both wild type and eed1Δ mutant cells during an experimental oral tissue infection using reconstituted human oral epithelium (RHE). Total fungal RNA was isolated 1 h, 12 h and 24 h after infection, labeled and hybridized to C. albicans DNA microarrays. Clustering of all transcriptomes during RHE infections revealed that wild type and eed1Δ transcript profiles clustered closely together at the earliest time point (1 h), reflecting the similar morphologies and pathogenic interactions with host cells (Fig. 3 A). However, at time points 12 and 24 h, profiles for the eed1Δ strain form a subcluster, which is more related to the 1 h time point of eed1Δ than to the transcriptional profiles of wild type cells at 12 and 24 h (Fig. 3 A). One hour after infection, only 59 genes were at least 2-fold up-regulated in wild type compared to eed1Δ, while 60 other genes were up-regulated in the mutant compared to the wild type (Fig. 3 B). Multiple genes associated with filamentous growth were similarly expressed in both wild type and eed1Δ, for example HWP1, CHT2 and ALS3 or the transcription factors CPH2, UME6 and TEC1 (Fig. 3 B, C). However, the global differences in expression between wild type and eed1Δ increased over time. Twelve h after infection, 548 genes were at least 2-fold differentially regulated in both strains (308 genes up-regulated in wild type, 240 up-regulated in eed1Δ, Fig. 3 B). This trend continued up to the 24 h time point. Out of 430 differentially regulated genes, 332 were up-regulated in wild type and only 98 were up-regulated in mutant cells (Fig. 3 B). No gene was up-regulated in the mutant at all time points, however, seven genes were always up-regulated in the wild type. Among these were the hyphae-associated genes ECE1 and HYR1 (Fig. 3 A, B). Other hyphal-associated genes, which were similarly expressed in wild type and mutant cells 1 h after infection, were down-regulated in the mutant at later time points, for example ALS3, HWP1 and SOD5 (Fig. 3 C). The pH response gene PHR1 was down-regulated in the mutant after 24 h (Fig. 3 C). Furthermore, the transcription factor genes CPR2, TEC1 (not shown) and UME6 or RD1, involved in polarized growth, were down-regulated in eed1Δ after 12 and 24 h (Fig. 3 C, Table S1), with UME6 being down-regulated 4-fold in the mutant after 24 h. Other genes, such as WOR2, involved in the regulatory circuit that controls white-opaque switching, were up-regulated in eed1Δ after 12 and 24 h (Table S2). Some genes associated with yeast-like growth were up-regulated in eed1Δ at later time points. Among these are the hyphal
growth repressor gene NRG1, which was 2.4-fold up-regulated in eed1Δ 24 h after infection (Fig. 3 C) and the amino acid permease gene AGP2 which is up-regulated in eed1D12 and 24 h after infection (Fig. 3 C, Table S2).

Expression of the regulatory gene UME6 depends on Eed1

Since eed1Δ cells showed a strong and significant down-regulation of UME6, recently shown to be necessary for the extension of germ tubes into hyphae [27], we focused further experiments on the genetic interactions between UME6 and EED1. In order to analyze the influence of EED1 on the expression level of UME6 in more detail, we quantified the expression of UME6 and monitored morphology during growth on plastic surfaces in a C. albicans wild type strain and an eed1Δ mutant strain carrying a pTET-EED1 construct at the ADH1 locus. After addition of 50 μg/ml doxycycline, which activated the tetracycline promoter driven EED1 allele, the mutant strain grew as filaments and expressed UME6 at significantly higher levels as compared to control cells in the absence of doxycycline (Fig. 4 A, B). pTET-driven expression of EED1 during infection of RHE restored filamentation and the ability to cause cell damage of the eed1Δ mutant (Fig. 4 C, D). These results indicate that expression of UME6 depends on the expression of EED1 and that the morphological defects of eed1Δ cells may be caused by a down-regulation of UME6 expression.

Ectopic overexpression of UME6 rescues filamentation in eed1Δ

Since UME6 was found to be down-regulated in eed1Δ, we hypothesized that UME6 may be a downstream target of Eed1 and that overexpression of UME6 in eed1Δ may rescue the lack of hyphal extension in eed1Δ. Therefore, we integrated a pTET-UME6 construct into the ADH1 locus of the eed1Δ mutant and analyzed the phenotype of eed1Δ after forced expression of UME6.

Growth of wild type, eed1Δ and eed1Δ + pTET-UME6 cells was tested under three different conditions: (a) growth on plastic, (b) growth under embedded conditions and (c) during infection of oral

Figure 3. Transcriptome analysis during experimental oral epithelial tissue infections. (A) Global clustering of transcriptional profiles of C. albicans wild type (WT) and eed1Δ during RHE infections over a time period of 24 h. (B) The numbers of genes differentially expressed at least with a 2-fold change are shown in Venn diagrams for all three points (1 h, 12 h, 24 h). (C) Dynamics of the expression of selected morphology-associated genes in WT and eed1Δ during the time course, shown by the ratio between expression in eed1Δ and expression in wild type.
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epithelial cells (TR-146 cell line). During growth on plastic and during co-incubation with host cells, wild type cells formed hyphae while the eed1Δ mutant grew as yeast cells after initial filamentation (Fig. 5). In contrast to the wild type, cells lacking Eed1 failed to produce any filaments under embedded conditions, showing that Eed1 is also required for filamentation under these conditions (Fig. 5). However, forced overexpression of UME6 within eed1Δ cells caused filamentation under all three conditions tested (Fig. 5).

Identification of Ume6-regulated gene subsets in eed1Δ

Since overexpression of UME6 in eed1Δ restored filamentation during growth on plastic surfaces, we analyzed the transcriptome of wild type, eed1Δ and eed1Δ+pTET-UME6 cells with or without doxycycline after 12 h growth on plastic (Fig. 6, Table S3 and S4) to identify genes regulated by Eed1 and/or Ume6. When comparing the transcriptome of wild type and eed1Δ cells, we identified 910 genes, which were at least 2-fold differentially expressed in the mutant. Of these, 441 were down-regulated and 469 were up-regulated in eed1Δ (Fig. 6, Table S3). Compared to eed1Δ, 313 genes were at least 2-fold differentially expressed after forced pTET-UME6 expression (Fig. 6, Table S4). Of these, 195 genes were up-regulated and 118 genes were down-regulated compared to eed1Δ (Fig. 6, Table S4). Clustering of gene expression pattern of wild type, eed1Δ and eed1Δ+pTET-UME6 cells showed that wild type and eed1Δ+pTET-UME6 cells showed similar morphologies (Fig. 5).

Among the 441 genes, which were down-regulated in the eed1Δ mutant on plastic (Table S3), were several hyphae-associated genes such as ECE1, HYR1, HWP1 and SOD5 and regulatory genes such as UME6 and HGC1 (Fig. 6B). All of them were also down-regulated in the eed1Δ mutant during RHE infections (Fig. 3). Ectopic overexpression of UME6 in eed1Δ could restore the expression of 117 of these 441 down-regulated genes to wild-type levels, including ECE1, HYR1, SOD5 and HGC1 and UME6 (Fig. 6B, Table S4). The remaining 324 genes did not reach wild type levels in the microarray analysis. However, 78 genes were expressed even higher in the eed1Δ mutant as compared to the wild type (Fig. 6B, Table S4). Among these were filament-associated genes like SAP6 and SUN41.

Among the 469 genes which were up-regulated in eed1Δ during growth on plastic as compared to the wild type were yeast-specific genes such as AGP2, regulatory genes like NRG1 and MIG1 and the adhesion genes ALS2 and ALS4 (Fig. 6C, Table S3). Some of these expression patterns (e.g. AGP2, NRG1) reflected the yeast cell growth of eed1Δ at the 12 h time point and were similar to the
However, only 82 out of the 469 genes up-regulated in eed1Δ were significantly down-regulated to wild type levels following ectopic overexpression of UME6 (Table S4), including ALS2, ALS4 and AQY1. The expression levels of 387 genes, including NRG1 and AGP2, remained unaffected by overexpression of UME6 in eed1Δ+ (Fig. 6 C). Thirty six genes exhibited greater down-regulation in eed1Δ+ pTET-UME6 as compared to wild type levels. Most of these genes were of unknown function including orf19.1780 and orf19.5503 (Fig. 6 C). These results show that a significant portion of EED1-regulated genes, including the most prominent hyphae-specific genes ECE1, SOD5 and HYR1, the cyclin gene HGC1 and the hyphal repressor gene NRG1, are regulated via UME6.

EED1 is up-regulated in C. albicans nrg1Δ and tup1Δ

In order to gain more information about the possible pathways which may be associated with EED1 regulation, we analyzed the expression of EED1 in mutants lacking key regulators of hyphal formation. The intergenic region between the open reading frame of EED1 and the 5’ upstream gene ITA6 is unusually long consisting of approximately 3 kb. Within this untranslated region we identified three putative Nrg1 response elements (NRE, Table S5,[21]), which suggest a possible regulation of EED1 expression by this repressor. In fact, under conditions which favor yeast growth of wild type cells (YPD, 30°C, Fig. 7 A), EED1 was significantly up-regulated in nrg1Δ cells as compared to the wild type (Fig. 7 B). Even higher up-regulation of EED1 was monitored in tup1Δ mutant cells at 30°C (Fig. 7 B). Both nrg1Δ and tup1Δ mutant cells grew as filaments under these conditions (Fig. 7 A). Under conditions that induce hyphal growth in wild type cells (RPMI 1640 medium, 37°C, Fig. 7 A), EED1 expression increased 10-fold in wild type hyphae compared to yeast cells (Fig. 7 B). In contrast, expression did not further increase and even slightly decreased in nrg1Δ mutant cells under conditions that induce hyphal growth in wild type cells (Fig. 7 B). Similar EED1 expression patterns were monitored in tup1Δ cells which reverted from hyphae to yeast cells and pseudohyphae (Fig. 7 A, B). These data suggest that EED1 is repressed by Nrg1 and Tup1 in wild type yeast cells.

EED1 expression depends on Efg1

In addition to NREs, the promoter of EED1 contains several E-box motifs which resemble binding sites of Efg1, the key
transcriptional regulator of the cAMP pathway (Table S5, [32]). A previous study has shown that expression of \( \text{EED1} \), formerly known as \( \text{EDT1} \) (Efg1-dependent transcript, [33]) was down-regulated in \( \text{efg1}^D \) mutant cells. In order to quantify expression of \( \text{EED1} \) in the \( \text{efg1}^D \) mutant, we incubated the \( \text{efg1}^D \) mutant and wild type cells under hyphal inducing conditions in liquid medium (RPMI) at 37°C. While the wild type formed hyphae under these conditions, \( \text{efg1}^D \) cells remained in the yeast-like growth phase. We found that \( \text{EED1} \) was approximately 10-fold down-regulated in \( \text{efg1}^D \) as compared to wild type hyphae (Fig. 7 B). In contrast, the expression level of \( \text{EED1} \) in \( \text{efg1}^D \) under conditions which favor yeast growth (YPD, 30°C, Fig. 7 A) was similar to wild type cells. These data suggest that \( \text{EED1} \) is a downstream target of Efg1.

**Ectopic overexpression of EED1 partially rescues filamentation in \( \text{efg1}^D \)**

If \( \text{EED1} \) is a crucial regulator of hyphal extension and a downstream target of Efg1, one would expect that a forced overexpression of \( \text{EED1} \) may cause filamentation in \( \text{efg1}^D \). To test this possibility, we ectopically integrated a \( \mu \text{TET-}\text{EED1} \) construct into the \( \text{ADH1} \) locus of the \( \text{efg1}^D \) mutant and studied morphology of the resulting mutant (\( \text{efg1}^D + \mu \text{TET-}\text{EED1} \)) with and without the addition of 50 \( \mu \)g/ml doxycycline during growth on plastic. As expected, wild type strains showed strong hyphal induction on plastic surfaces and \( \text{efg1}^D + \mu \text{TET-}\text{EED1} \) failed to form filaments without the addition of doxycycline (Fig. 8 A). Induction of \( \text{EED1} \) by the addition of doxycycline in \( \text{efg1}^D + \mu \text{TET-}\text{EED1} \) caused...
formation of hyphae or pseudohyphae in approximately 80% of all cells after 12 h (Fig. 8 A). Since filamentation of efg1 Δ mutant cells expressing EED1 was observed, we analyzed the expression of the hyphal-associated genes ECE1 and HWP1 and the hyphal regulatory gene UME6 in wild type, efg1 Δ and efg1 Δ + pTET-EED1 cells. ECE1 and HWP1 were approximately 10-fold down-regulated in efg1 Δ as compared to the wild type (Fig. 8 B). Forced expression of EED1 in efg1 Δ + pTET-EED1 caused increased expression of HWP1 similar to wild type levels (Fig. 8 B). Similarly, expression of ECE1 was increased in efg1 Δ + pTET-EED1, although expression levels were slightly lower as compared to the wild type (Fig. 8 B). For UME6, we observed 5-fold reduced expression in efg1 Δ + pTET-EED1 cells without addition of doxycycline as compared to the wild type (Fig. 8 B). However, during forced expression of pTET-EED1 by the addition of doxycycline, the expression of UME6 increased to the level of wild type hyphae (Fig. 8 B). This supports the hypothesis that UME6 expression depends on Eed1. Confirming previous observations made by Zeidler et al. [28], we also observed that forced expression of UME6 restored filamentation in efg1 Δ cells (Fig. 8 C). It should be noted that hyphal growth in efg1 Δ + pTET-UME6 seemed to be stronger than in efg1 Δ + pTET-EED1 after addition of doxycycline (Fig. 8 A, C). Taken together, these data suggest that forced expression of EED1 not only triggered hyphal formation, but also expression of hyphal-associated genes.

**Discussion**

**EED1 is a unique, species-specific gene of C. albicans**

Although the gene locus of EED1 is conserved within the ascomycetes CUG clade, we did not find a homologous gene in any genome sequence accessible via NCBI (http://bla. b.ncbi.nlm.nih.gov/Blast.cgi). Only C. dubliniensis contains a syntetic gene at the same locus named MDP1 (Moran et al., unpublished data). However, the overall homology between Eed1 and Mdp1 is low and functional analysis of Mdp1 in C. dubliniensis suggests different roles of both proteins (Moran et al., unpublished data). Therefore, we concluded that EED1 is a unique gene.

The only putative motif of the deduced protein of EED1, which may indicate a cellular function, is a central glutamine-rich region similar to the *S. cerevisiae* protein Def1 [12,29,30], which may facilitate interactions with other proteins. In *C. albicans*, many regulatory proteins including Efg1, Cph1, Ume6 and Tec1 possess such multi glutamine stretches (http://www.
Eed1 is a key factor within the network regulating dimorphism

Due to the importance of C. albicans morphology for pathogenicity, multiple studies have investigated the processes involved in hyphal formation and several pathways regulating the morphological transition from yeast-to-hyphal cells have been described [14,15,22]. However, the regulation of hyphal extension of primary filaments into long and dividing hyphae is less well studied. Due to the phenotype of cells lacking EED1, which were able to form germ tubes but failed to extend these into hyphae with dramatic consequences during infection of epithelial tissues [12], we have focused our study (1) on the regulation of hyphal extension and (2) the gene expression associated with hyphal extension. The results of our work dealing with the regulatory role of Eed1 during dimorphism were combined with recent findings of other studies and summarized in a model shown in Fig. 9. In this model, C. albicans cells grow in the yeast form until an external stimulus triggers the formation of germ tubes. This step includes the well-studied MAP kinase cascade and the cAMP pathway as discussed in several reviews [14,15,22]. Key regulators of germ tube formation include the GTPase Ras1 and the transcription factors Cph1 and Efg1 (Fig. 9). However, a second regulatory network is required to promote extension of germ tubes into hyphae. When this regulation fails, germ tubes switch back to yeast cells as shown for mutants lacking Eed1 or Ume6 in [12,27] and in this work (Fig. 9). We propose that Eed1 is the primary element of a regulatory cycle which controls hyphal extension on a transcriptional level. As shown in this work, EEDI expression depends on Efg1, a member of the APSES gene family [37] (Fig. 9), confirming previous data of the Fink group who originally named EED1 (orf19.7561) EDT1 [33]. Expression of EED1 is also regulated by Nrg1 as shown by transcriptional profiling of the nrg1Δ mutant [37] and quantitative RT-PCR (this work) (Fig. 9). The second step of the hyphal extension cycle is an EEDI-dependent expression of UME6 (Fig. 9). In this work we provide evidence that expression of UME6 depends on the expression levels of EEDI, in particular, mutants lacking either EED1 or UME6 share very similar phenotypes [12,27], supporting our hypothesis that both have important roles in hyphal extension. Similar to EEDI, UME6 is repressed by Nrg1 [27,38] (Fig. 9). It is unclear whether Efg1 directly activates UME6 expression. We show that UME6 was down-regulated in efg1Δ mutant cells,
however, expression of \textit{UME6} was restored by forced overexpression of \textit{EED1}. We suggest that \textit{EED1}-dependent \textit{UME6} expression is an essential step of hyphal extension in \textit{C. albicans}. Overexpression of \textit{UME6} in \textit{eed1Δ} not only restored filamentation, but also expression of prominent hyphal-associated genes like \textit{ECE1}, \textit{HWP1} and \textit{HMR1}. Previously it was shown that \textit{UME6} expression levels have an impact on the growth of \textit{C. albicans} as either pseudohyphae (low \textit{UME6} expression levels have an impact on the growth of \textit{ECE1} like \textit{HWP1}) but also expression of prominent hyphal-associated genes. Interestingly, \textit{Ume6} seems also to be involved in keeping lower expression levels low in \textit{NRG1} expression [39]. High expression of \textit{UME6} correlated with higher expression of hyphal-associated genes such as \textit{ECE1} and \textit{HWP1} [40], which is supported by our findings. Interestingly, \textit{Ume6} seems also to be involved in keeping low expression levels low in \textit{C. albicans} hyphae [27]. As both, \textit{EED1} and \textit{UME6}, are repressed by \textit{Nrg1}, this mechanism could contribute to regulating hyphal extension. Our transcriptional data show an up-regulation of \textit{NRG1} in \textit{eed1Δ} during both, infection of \textit{RHE} and growth on plastic, and this up-regulation was reversed by ectopic overexpression of \textit{UME6} in \textit{eed1Δ}. This may indicate that \textit{Eed1}-dependent up-regulation of \textit{UME6} is required to keep \textit{NRG1} expression at low levels in order to prevent \textit{Nrg1}-mediated reversion of hyphae to yeast cells. Another important target of \textit{Ume6} seems to be the \textit{HGC1} gene, encoding a hypha-specific G1 cyclin [23,40] [Fig. 9]. \textit{HGC1} was down-regulated in \textit{eed1Δ} at late phases of \textit{RHE} infections and growth on plastic. This down-regulation was bypassed by ectopic overexpression of \textit{UME6} in \textit{eed1Δ}, indicating that \textit{HGC1} belongs to the set of genes which is regulated by \textit{Ume6} as reported previously [40]. \textit{Hgc1} itself, together with its interaction partner \textit{Cdc28}, is involved in the phosphorylation of \textit{Efg1} [25], \textit{Cdc42} activation [24] and the regulation of polarized secretion [41]. It should be noted, that during growth on plastic, \textit{CDC28} was down-regulated in \textit{eed1Δ} and strongly up-regulated in \textit{eed1Δ} overexpressing \textit{UME6} (Fig. 6). A previous report has shown that mutants lacking another G1 cyclin, \textit{Ccnc1}, were not able to maintain filamentous growth although these cells produced initial germ tubes similar to \textit{eed1Δ} and \textit{ume6Δ} mutant cells [42]. However, in our experiments we did not observe significant changes of \textit{CCN1} expression levels in \textit{eed1Δ} cells suggesting no direct link between \textit{Eed1} and \textit{Ccnc1}. Other genes encoding factors involved in polarized growth, including \textit{CDC42}, \textit{RDI1}, \textit{MYO2}, \textit{CDC11}, \textit{CIB2}, \textit{MOB1} and \textit{MLC1} were down-regulated in the \textit{eed1Δ} mutant. Their expression was partially restored to wild type levels after forced overexpression of \textit{UME6} (supplemental tables S3 and S4). These results provide a link between the transcriptional regulation of hyphal extension presented here (Fig. 9) and the known cellular requirements for maintenance of polarized growth and associated structures such as the spitsenkörper. This may explain why mutant cells lacking key elements of the hyphal extension cycle lose their ability to maintain polarized hyphal growth and therefore switch from germ tubes to yeast cell growth. Analysis of the transcriptome data during growth on plastic also showed a slight up-regulation of \textit{PES1} in the \textit{eed1Δ} mutant. This gene was reported to encode a regulator of the hypha-to-yeast switch in \textit{C. albicans} [26] and also plays an important role for dispersion of yeast cells from biofilms [43]. The up-regulation of \textit{PES1} was partially reverted to wild type levels in \textit{eed1Δ} overexpressing \textit{UME6}. This further supports the view that \textit{Eed1} and \textit{Ume6} are involved in the repression of the reversion of hypha into yeast cells. It was already shown that \textit{Ume6} might have a negative influence on dispersion of yeast cells from biofilms [43,44]. Therefore, it may be postulated that \textit{Eed1} also plays a role during biofilm formation.

In summary, our data suggest that \textit{Eed1} and \textit{Ume6} act in a pathway regulating the maintenance of hyphal growth thereby repressing the hyphal-to-yeast transition.
Materials and Methods

**In silico analysis**

The search for homologs of *C. albicans* Eed1 was performed with protein Blast and (Blast from the NCBI home page (http://blast.ncbi.nlm.nih.gov/Blast.cgi). To compare synteny for the EED1 locus within the CUG Candida clade we have used the online databases from the Broad Institute (http://www.broadinstitute.org/annotation/genome/candida_group/MultiHome.html) and from the Sanger Institute (http://www.sanger.ac.uk/Projects/Fungi/). Sequences were aligned with DNASTAR Lasergene MegAlign software.

**Strains and media**

*C. albicans* strains (listed in Table 1) were routinely grown in YPD medium (1% yeast extract, 2% bacto-peptone, 2% D-glucose) at 30°C or 37°C in a shaking incubator overnight. Prior to use in experiments, fungal cells were semisynchronised by incubating twice in YPD overnight and washed three times with 1x PBS. Cells were counted with a Neubauer chamber and added to experimental assays at the given concentrations. For growth under embedded conditions, cells were grown overnight in YPD medium at 30°C, washed with 1x PBS, diluted to a concentration of 1x10⁶ cells/ml and mixed with YPS agar (1% yeast extract, 2% bacto-peptone, 1% agar, 2% sucrose), plated and incubated for 3 days at 25°C (modified from [45]). Colonies were analyzed with a Leica DM II inverted microscope (Leica Microsystems, Wetzlar, Germany). Image acquisition and analysis was done with the Leica Application Suite Software.

**Cell lines and cell culture**

In this study we have used the human oral epithelial cell line TR146 [46]. TR146 cells were grown in DMEM medium with 10% FBS at 37°C and 5% CO₂ until they have reached confluency. For infection assays, 1x10⁶/ml human cells were plated into 24 well plates and grown in DMEM + 10% FBS at 37°C and 5% CO₂ until confluency was reached. Prior to infection, cells were washed and then FBS-free DMEM was added. For infection, *C. albicans* strains were grown in YPD at 30°C overnight and then diluted to OD₆₀₀ = 0.2 in fresh YPD, followed by a new incubation at 30°C for approximately 4 h. Cells were harvested by centrifugation, washed three times with 1x PBS and finally resuspended in 1x PBS. Host cells were infected with fungal cells in different cell numbers ranging from 5x10⁶ (24 h infection) to 1x10⁷ cells (3 h infection). Infection assays were incubated at 37°C and 5% CO₂ for a maximum of 24 h.

**Experimental oral epithelial tissue infection**

For the analysis of invasion abilities of different *C. albicans* strains we have used the reconstituted human oral epithelial (RHE, SkinEthic, Nice, France) which consists of differentiated multilayers of the TR146 cell line. Infection assays were performed as described previously [47]. Prior to infection, *C. albicans* strains were grown in YPD at 30°C overnight and then diluted to OD₆₀₀ = 0.2 in fresh YPD, followed by a new incubation at 30°C for approximately 4 h. Cells were harvested by centrifugation, washed three times with 1x PBS and finally resuspended in 1x PBS. 2x10⁶ *C. albicans* cells were added to each RHE. The infection assays were incubated for a maximum of 24 h at 37°C and 5% CO₂. 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 490 nm using a Genios plate reader ( Tecan UK Ltd.). One unit of LDH activity is equivalent to 1 μM formazan formed per reaction. Result shown were generated from three separate infections. Prior to sectioning and staining for light microscopy, RHE tissues were fixed in 4% (v/v) paraformaldehyde in PBS (pH 7.4), dehydrated in ethanol and embedded in paraffin wax. Sections were stained with Periodic Acid Schiff (PAS) reagent for visualization of fungal elements. Tissues were examined using a Nikon Eclipse 600 microscope.

**Construction of *C. albicans* mutants**

For transformation of *C. albicans* we have used the lithium-acetate method as previously described [48]. Ura− mutant strains were recovered by integrating the plasmid pCIP10 (URA4) into the RPS10 locus [49,50]. A parental strain was created by integrating pCIP50 (URA4; HIS1; ARG6) [51] into the RPS10 locus of BWP17. pCIP10 and pCIP30 were kindly provided by A. Brown, Aberdeen. The pTET-UME6 construct was excised with the restriction enzymes Apal and PstI from a pNIM1 derivative kindly provided by A. Bito, University of Salzburg [28] and transformed into *eed1Δ* mutant strains. For experiments including *eed1Δ* we have used the reconstituted human oral epithelium (RHE, SkinEthic, Nice, France). To compare synteny for the EED1 locus within the CUG Candida clade we have used the online databases from the Broad Institute (http://www.broadinstitute.org/annotation/genome/candida_group/MultiHome.html) and from the Sanger Institute (http://www.sanger.ac.uk/Projects/Fungi/). Sequences were aligned with DNASTAR Lasergene MegAlign software.

| Table 1. *Candida albicans* strains used in this study. |
|---------------------------------|
| **Strain** | **Genotype** | **Reference** |
| SC3514 | *C. albicans* wild type | [56] |
| BWP17 + CIP30 | *ura4Δ::imm434/ura3Δ::imm434 his4::hisG1his1::hisG arg4::arg4::his4::hisG plus pCIP30 | [12] |
| HLC52 (eed1Δ) | eed1::hisG/eed1::HisG-UA3-URA3-HIS1 in CAI-4 | [16] |
| MMC4 (ng1Δ) | ng1::hisG/ng1::hisG in CAI-4 | [21] |
| BCA 2-10 (tup1Δ) | tup1::hisG/tup1::hisG in CAI-4 | [20] |
| UZ149 (pTET-UME6) | ADH1/adt1::SAT1-pTET-UME6 in SC5314 | [28] |
| M1263 (eed1Δ) | eed1::HIS1/eed1::ARG4, pCIP10 in BWP17 | [12] |
| M1273 (eed1Δ + EED1) | EED1/EED1, pCIP30 plus pCIP10 | [12] |
| M1315 (eed1Δ) | eed1::FRT/eed1::FRT in SC5314 | [12] |
| M1457 (tup1Δ) | BCA 2-10 plus pCIP10 | this work |
| M1458 (ng1Δ) | MMC4 plus pCIP10 | this work |
| M1563 (eed1Δ + pTET-UME6) | ADH1/adt1::SAT1-pTET-UME6 in M1263 | this work |
| M1573 (eed1Δ + pTET-EED1) | ADH1/adt1::SAT1-pTET-EED1 in HLC52 | this work |
| M1574 (eed1Δ + pTET-EED1) | ADH1/adt1::SAT1-pTET-EED1 in HLC52 | this work |
| M1764 (eed1Δ + pTET-UME6) | ADH1/adt1::SAT1-pTET-UME6 in HLC52 | this work |
| M1773 (EED1/eed1) | EED1/eed1::ARG4, ura3, his1, pCIP30 in BWP17 | this work |
| M1774 (eed1/eed1ΔN) | eed1::ARG4/EED1ΔN::HIS1, ura3, pCIP10 in BWP17 | this work |
| M1775 (eed1/eed1ΔNQ) | eed1::ARG4/EED1ΔNQ::HIS1, ura3, pCIP10 in BWP17 | this work |
| M1776 (eed1/eed1ΔQC) | eed1::ARG4/EED1ΔQC::HIS1, ura3, pCIP10 in BWP17 | this work |
| M1777 (eed1/eed1ΔC) | eed1::ARG4/EED1ΔC::HIS1, ura3, pCIP10 in BWP17 | this work |

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we have amplified the SAT1+pTET fragment from pNIM1 and the EED1 ORF from genomic C. albicans SC5314 DNA with overhanging oligonucleotide primers (see Table S6). Both PCR fragments were linked in a fusion PCR and transformed into C. albicans. pTET-UME6 or pTET-EED1 were integrated into the ADH1 locus as described previously [52]. Transformants were selected on YPD with 200 μg/ml nourseothricin [53] and were verified by PCR and Southern Blot analysis. All oligonucleotide primers used in this study are listed in supplemental table S6.

RNA isolation

For RNA isolation cells were harvested by centrifugation and resuspended in 400 μl AE buffer (50 mM sodium acetate, 10 mM EDTA). Next, 40 μl 10% SDS and an equal volume of phenol/chloroform/isoamylalcohol was added. Mixtures were incubated at 65°C for 5 min, followed by an incubation at −80°C until they were frozen. After a second incubation at 65°C (until samples were thawed) the mixtures were centrifugated for 2 min at 12000xg. The upper liquid phase was transferred into a new reaction tube. After addition of 10% volume sodium acetate pH 5.3 and 1 volume 2-propanol, RNA was precipitated for 30 min at −20°C. Samples were centrifugated for 10 min at 12000xg, supernatant were discarded and RNA pellets washed twice with 70% ethanol (prepared with RNase free water). Finally, RNA was solved in RNase free water. Qualities and quantities of isolated RNA were analyzed with an Agilent 2100 Bioanalyzer (Agilent Technologies).

Transcriptional profiling

For transcriptional profiling we have used C. albicans oligo microarrays (Eurogentec, Seraing, Belgium). Sample RNA from RHE and plastic assays was labelled with Cy5 (GE Healthcare). These sample RNAs were cohybridized with a common reference (RNA from SC5314 grown in YPD, mid-log phase, 37°C, Cy3-labelled). Slides were hybridized, washed and scanned with Genepix as described [54]. Data normalization (LOWESS) and analysis was performed with Genespring 7.2 software (Agilent Technologies) as described previously [12,54]. Microarray studies were done in three biological independent triplicates. Student's t-test was used to compare expression data from the triplicates to identify significant differences. Only differences with p<0.05 were used as statistically significant. All microarray data are MIAME compliant and raw data have been deposited at ArrayExpress (http://www.ebi.ac.uk/microarray-as/ae). The accession numbers are E-MEXP-3085 for the RHE infection microarrays and E-MEXP-3083 for the plastic growth experiments.

Quantitative RT PCR

For gene expression analysis, 100 ng of total RNA were used to perform quantitative RT-PCR with the One Step RT-qPCR Master Mix Plus for SYBR Green I Kit (Eurogentec, Seraing, Belgium). RT-PCR was performed on a Applied Biosystems 7300 Fast Real-Time PCR System (Applied Biosystems, Darmstadt, Germany). Expression was calculated by the ΔΔCt method as described previously [55]. Student’s t-test was used to compare expression data from the triplicates to identify significant differences. Only with p≤0.05 differences were regarded as statistically significant.

Growth on plastic surfaces

Strains were twice grown in YPD at 30°C overnight for semisynchronisation. Cells were washed with 1x PBS and 1×10^6 cells/ml were added to RPMI1640 medium (PAA). This mixture was given on petri dishes or 6-well plates and incubated for 24 h at 37°C at 5% CO2 in the air. For microscopy, a Leica DM IL inverted microscope (Leica Microsystems, Wetzlar, Germany) was used. To analyse dynamics of growth on plastic via timelapse microscopy we have used the same environmental conditions and a Zeiss AxioObserver. Z1 fluorescence microscope (Zeiss, Gottingen).

Microscopy

Differential staining of C. albicans during infection of host cells was done as described previously [9]. After fixation and washing with 1x PBS C. albicans cells were stained with rabbit anti-C. albicans polyclonal antibody conjugated with Alexa Fluor 560 (Invitrogen). Subsequently, human host cells were permeabilised with 0.5% Triton X-100 and Calcofluor White was added to stain C. albicans cells. Therefore, fungal elements of C. albicans outside of host cells were stained with the antibody and Calcofluor White while parts within the host cells were stained with Calcofluor White only. For staining of cells grown on glass coverslips without human cells, C. albicans cells were fixed with 4% histofix for 30 min (Carl Roth, Karlsruhe, Germany), washed with PBS and stained with Calcofluor White prior to microscopy. Microscopy was performed with a Leica DM 5500B fluorescence microscope (Leica Microsystems, Wetzlar, Germany). Image acquisition and analysis was done using the Leica Application Suite Software.

Supporting Information

Movie S1 SC5314 RPMI plastic 37°C 5% CO2 (AVI)
Movie S2 eed1 RPMI plastic 37°C 5% CO2 (AVI)
Table S1 Genes down-regulated in eed1 during RHE infection (XLS)
Table S2 Genes up-regulated in eed1 during RHE infection (XLS)
Table S3 Transcriptome plastic growth WT vs eed (XLS)
Table S4 Transcriptome plastic growth eed1+pTET-UME6 vs eed (XLS)
Table S5 Binding motifs in intergenic region upstream of EED1 (DOC)
Table S6 Oligonucleotide primers used in this study (DOC)

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Author Contributions

Conceived and designed the experiments: RM GPM IDJ AH JD. Analyzed the data: RM GPM IDJ DJS OK BH. Performed the experiments: RM GPM IDJ DJS OK BH. Wrote the paper: RM GPM IDJ DJS OK BH.
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