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A *Candida albicans* cell wall-linked protein promotes invasive filamentation into semi-solid medium

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SUMMARY

Growth of cells in contact with an abiotic or biological surface profoundly affects cellular physiology. In the opportunistic human pathogen, *Candida albicans*, growth on a semi-solid matrix such as agar results in invasive filamentation, a process in which cells change their morphology to highly elongated filamentous hyphae that grow into the matrix. We hypothesized that a plasma membrane receptor-type protein would sense the presence of matrix and activate a signal transduction cascade, thus promoting invasive filamentation. In this communication, we demonstrate that during growth in contact with a semi-solid surface, activation of a MAP kinase, Cek1p, is promoted, in part, by a plasma membrane protein termed Dfi1p and results in invasive filamentation. A *C. albicans* mutant lacking Dfi1p showed reduced virulence in a murine model of disseminated candidiasis. Dfi1p is a relatively small, integral membrane protein that localizes to the plasma membrane. Some Dfi1p molecules become cross-linked to the carbohydrate polymers of the cell wall. Thus, Dfi1p is capable of linking the cell wall to the plasma membrane and cytoplasm.

Keywords

Candida albicans; invasive filamentation; cell wall; plasma membrane; MAP kinase

INTRODUCTION

Cells that grow in contact with a surface have a different physiology and metabolism than their liquid-grown or detached counterparts (Ingber, 2006, Kumamoto & Vinces, 2005). Within the fungal kingdom, there exist many examples of contact-dependent behavior (Tucker & Talbot, 2001, Kumamoto & Vinces, 2005). Many fungal plant pathogens germinate only after they attach to a surface (Ahn *et al.*, 2003, Doehlemann *et al.*, 2006, Kuo & Hoch, 1996). In addition, the ability to adjust polarized growth to physical cues present in the substratum, thigmotropism, is observed in fungi as well as plant roots and neurons (Gow, 1997, Migliaccio & Piconese, 2001). Invasive growth, in which organisms respond to growth on a semi-solid surface by growing into the surface, is observed in the human commensal and opportunistic pathogen *Candida albicans* (Kumamoto & Vinces, 2005), and the model organism *Saccharomyces cerevisiae* (Gimeno *et al.*, 1992, Gancedo, 2001).

As a human commensal, *C. albicans* resides on mucosal surfaces such as the tongue, gastrointestinal and genitourinary tracts (Odds, 1987, Soll *et al.*, 1991). Immunocompromised hosts are susceptible to superficial *C. albicans* mucosal infections

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such as thrush or vaginitis, and to life-threatening disseminated disease. In a compromised host, *C. albicans* produces characteristic invasive lesions in which filamentous cells, either hyphae or pseudohyphae (Sudbery *et al.*, 2004, Berman, 2006), invade the tissue of the host. This behavior allows the organism to breach epithelial barriers and reach the blood stream, resulting in disseminated disease.

In the laboratory, *C. albicans* cells respond to growth in contact with agar medium by producing filaments that invade the agar. Production of invasive hyphae during growth in laboratory medium may occur by the same mechanism that is involved in production of invasive lesions during candidiasis. How fungal cells sense that they are growing on an agar matrix and respond by producing invasive filaments is not well understood. However, signaling events occurring in such cells have been detected. In response to growth on an agar surface, *C. albicans* cells activate a mitogen activated protein kinase (MAPK) of the ERK1/2 (Extracellularly Regulated Kinase) superfamily called Mkc1p (Kumamoto, 2005). Among plant pathogens, homologs of Mkc1p and a second MAPK are necessary for tissue invasion and pathogenesis (Doehlemann et al., 2006). Therefore, the goal of the present study was to identify a *C. albicans* plasma membrane protein important for initiation of invasive filamentation and matrix-dependent MAPK signaling.

Here we describe a gene, Orf19.7084, renamed *DFI1* (Defective in Filamentous Invasion 1), which encodes a cell surface glycoprotein that promotes matrix-dependent activation of Cek1p. Dfi1p is also required for full *C.* albicans virulence in the intravenously inoculated mouse model of disseminated candidiasis. A glycine-rich transmembrane segment containing a GxxxG motif, similar to the dimerization motif found in the mammalian red blood cell protein glycophorin A (Smith *et al.*, 2001, Brosig & Langosch, 1998), is important for Dfi1p function. A subpopulation of Dfi1p is covalently bound to the cell wall, allowing Dfi1p to monitor events affecting the cell wall. Initiation of invasive filamentation may thus involve responses to events that alter the properties of the carbohydrate-rich cell wall.

RESULTS

Signal transduction pathways activated during growth on a semi-solid surface

The MAPK Mkc1p is activated during growth of *C. albicans* on agar matrix (Fig. 1A, P-M) (Kumamoto, 2005). To identify other MAPKs that are similarly activated, extracts of cells grown in liquid medium (YPS) were compared to extracts of cells grown on the surface of agar medium (YPSA) by Western blotting with antiserum that detects dually-phosphorylated forms of ERK1/2 superfamily MAPKs. Activation of a second MAPK was detected in extracts of surface-grown cells at higher levels than in liquid-grown cells (Fig. 1A, P-C). The electrophoretic mobility of this MAPK was consistent with the molecular weight of the MAPK Cek1p (49kDa) (Fig. 1A, third panel). Extracts of strain CCC55 (*cek1* null mutant; (Csank et al., 1998)) yielded no 49kDa signal with either anti-phospho-MAPK or anti-Cek1p antiserum (Fig. 1A, lane 1). Furthermore, extracts of surface-grown cells of strain CCC81, which lack a phosphatase thought to act on phospho-Cek1p (Csank et al., 1997), showed increased amounts of phospho-Cek1p (Fig. 1A, lane 2). Therefore, Cek1p is activated during growth on the surface of agar matrix.

Previous studies showed that a mutant lacking Cek1p is defective in filamentation during growth on the surface of several types of agar media such as mannitol-containing Lee's medium, Spider medium or low ammonia medium (Csank et al., 1998). In addition, when grown on the surface of YPSA, CCC55 cells (*cek1* null mutant) failed to adhere to the agar and did not produce invasive filaments; when embedded within YPS agar, the mutant was delayed in producing filaments (data not shown). In contrast, strain CCC81, lacking the

phosphatase Cpp1p, is hyperinvasive when grown under non-hypha inducing conditions, such as growth on agar medium at 25°C (Csank et al., 1997). These findings argue that activation of Cek1p promotes adhesion to an agar surface and invasion but that filamentation when embedded in agar can occur in the absence of Cek1p.

A gene required for C. albicans invasive filamentation

To understand events that lead to activation of MAPKs and invasion of a semi-solid material, we sought to identify a plasma membrane protein that initiates the signaling for matrix-dependent activation of Mkc1p or Cek1p. Several *C. albicans* candidate genes encoding putative membrane proteins were deleted (Table 1). Four candidate genes encoded signaling proteins or were homologous to *S. cerevisiae* proteins involved in activation of MAPKs (orf19.4772, orf19.1490, orf19.5867, orf19.5537) (Roman *et al.*, 2009, Roman *et al.*, 2005). Four candidate genes lacked clear orthologs in *S. cerevisiae* and were chosen on the basis of predicted structure or presence of motifs (orf19.7084, orf19.207, orf19.4906 and orf19.1488). Three independent null mutants were generated for each gene tested. Invasive filamentation when embedded or plated on the surface of agar medium, and growth in the presence of the cell wall active agents Congo Red and Calcofluor White were tested.

Mutants lacking orf19.7084 exhibited defects in filamentation during embedded growth (Fig. 2; Table 1); mutants lacking Ca*SHO1* (orf19.4772), or Ca*MSB2* (orf19.1490), were delayed in producing filaments under these conditions (Fig. 2; Table 1). The other mutants were not defective in invasive filamentation (Fig. 2; Table 1). The rest of this report will focus on orf19.7084 (termed *DFI1* for Defective in Filamentous Invasion). *DFI1* is predicted to encode a 36.5kDa transmembrane protein with a hydrophobic N-terminal region, a large, extracellular, serine and threonine-rich portion, a second hydrophobic region and a short C-terminal tail. Orthologs of Dfi1p are encoded in the genomes of a few other fungi such as *Candida tropicalis, Lodderomyces elongisporus* and *Debaryomyces hansenii* but are missing from more distantly related fungi such as *S. cerevisiae*, plants or animals. Similarities to the Pfam HMM Mid2 protein family (30% identity over the C-terminal 35% of Dfi1p) and to the SKG6 transmembrane alpha-helix domain (39% identity over the transmembrane and juxtamembrane regions) were detected. Similarities to BLOCKS from carbohydrate-binding WSC proteins and flocculins, and motifs for N-linked glycosylation were also found.

dfi1 null mutants are defective in invasion of agar medium

When grown on the surface of YPSA at either 25°C or 37°C, cells of the WT strain adhered to the surface and invaded the agar (Fig. 3A). Under these conditions, the *dfi1* null mutant was deficient in adhering to and invading the agar (Fig. 3A). Similarly, when the strains were grown embedded in agar medium at 25°C or 37°C, the WT strain produced invasive filaments but the *dfi1* null mutant failed to do so (Fig. 3B and data not shown).

However, *dfi1* null mutants were not defective in growth rate (e.g. in YPD liquid medium at 30°C, doubling times for WT and the *dfi1* null mutant were 2.0 hours). The heterozygous *DFI1/dfi1* strain behaved like the WT parent strain (data not shown). Introduction of either a wild-type, untagged *DFI1* allele or tagged *DFI1* alleles at the *DFI1* locus restored agar invasion and filamentation under embedded conditions, demonstrating that the defect was due to the lack of Dfi1p (Fig. 3A, B, C).

In YPD liquid medium supplemented with serum (10%; Fig. 3D), Spider liquid medium (data not shown) or RPMI liquid medium (data not shown) at 37°C, *dfi1* null mutants germinated and produced elongated hyphae to the same extent as wild type cells, demonstrating that *dfi1* null mutants retained the ability to produce hyphae. The *dfi1* null

mutant also adhered to polystyrene and germinated normally under biofilm forming conditions (data not shown). Thus, cells lacking Dfi1p failed to produce filaments in response to growth in contact with agar matrix, but were not inherently unable to form filaments.

Dfi1p function promotes matrix-dependent activation of Cek1p kinase

To determine the effect of Dfi1p on activation of MAPKs, extracts were made from WT or *dfi1* null mutants grown in liquid medium or on the surface of agar. As shown in Fig. 1A, extracts obtained from surface-grown *dfi1* null cells (Fig. 1A, lane 6) contained lower than WT levels of phospho-Cek1p (Fig. 1A, lane 5) but levels of phospho-Mkc1p were unchanged. In liquid medium neither wild type nor *dfi1* null mutant strains contained high levels of activated Cek1p or Mkc1p (Fig. 1A, lanes 3, 4). Low levels of Cek1p activation were observed in liquid cultures in exponential phase, or early or late post-exponential phase (data not shown), consistent with previous observations (Roman et al., 2009). Levels of total Cek1p (Fig. 1A, third panel) and actin (Fig. 1A, bottom panel) were comparable in all samples. The defect in activation of Cek1p was complemented by introduction of a WT copy of *DFI1* into the *dfi1* null mutant (Fig. 1B). Therefore, the *dfi1* null mutant failed to activate Cek1p to WT levels in response to growth on an agar surface.

Increasing levels of phospho-Cek1p in a dfi1 null mutant restores invasive filamentation

During growth on the surface of agar medium, Cek1p is not activated to normal levels in the absence of Dfi1p. If this reduction in phospho-Cek1p level is important, then increasing the amount of activated Cek1p in a *dfi1* null mutant might restore agar invasion. Since the phosphatase Cpp1p dephosphorylates phospho-Cek1p ((Csank et al., 1997) and Fig. 1A), reducing the gene dosage of *CPP1* should increase phospho-Cek1p levels. Therefore, one allele of *CPP1* phosphatase was deleted in wild type or *dfi1* null mutant strains. As shown in Fig. 4A, during growth on agar medium, strain pcz6 (*dfi1/dfi1, CPP1/cpp1*) accumulated higher levels of phospho-Cek1p than the *dfi1* null strain. The amount of phospho-Mkc1p signal was much higher than the phospho-Cek1p signal and did not change substantially in different strains (Fig. 4B).

When grown on the surface of YPSA medium, cells of strain pcz6 (*dfi1/dfi1, CPP1/cpp1*) adhered to the surface and invaded the agar (Fig. 4C). When embedded in YPSA, strain pcz6 (*dfi1/dfi1, CPP1/cpp1*) produced filaments at levels resembling the wild type strain (Fig. 4D). Therefore, increased invasive filamentation accompanied an increased level of phospho-Cek1p. Furthermore, the *CPP1/cpp1* heterozygote strain was hyperinvasive when compared to the wild type strain, in agreement with the literature (Csank et al., 1997). These results support the conclusion that activation of Cek1p due to the activity of Dfi1p promotes adhesion and invasive filamentation in response to growth in contact with semi-solid material.

A GxxxG Glycophorin A motif in the transmembrane domain of Dfi1p is important for Dfi1p function

The putative transmembrane domain (TMD) of Dfi1p contains a GxxxG motif (Fig. 5A) that is very similar to the GxxxG dimerization motif first described in glycophorin A (Brosig & Langosch, 1998), and later observed in numerous transmembrane proteins (Smith et al., 2001, Curran & Engelman, 2003). To determine whether this motif was important for Dfi1p function, a mutant allele in which glycine 273 and glycine 277 were replaced with leucines (*dfi1*$_{G273,277L}$) was constructed. When grown on the surface of YPSA medium, a strain that expressed mutant Dfi1$_{G273,277L}$p-TAP as the sole form of Dfi1p failed to adhere to the agar or to produce filaments that invaded the surface (Fig. 5C). The mutant was also defective in invasive filamentation when grown embedded within agar medium (Fig. 5D).

In addition, the *dfi1*$_{G273,277L}$ mutant failed to activate Cek1p to wild type levels when grown on an agar surface (Fig. 5B). The mutant protein was expressed and localized normally (discussed below). Therefore, the GXXXG motif is important for the ability of Dfi1p to promote Cek1p activation and invasive filamentation during growth on an agar surface, suggesting that multimerization is important for the function of Dfi1p.

Localization and processing of Dfi1p

The *DFI1* gene is predicted to encode a 36.5kDa glycosylated protein (both O- and N-linked glycosylation) with two hydrophobic domains. To determine the localization of the protein, *DFI1* was tagged at the C terminus with GFP or HA and the tagged proteins were analyzed. All tagged alleles were under the control of the *DFI1* promoter, retained their native 3' untranslated regions, and yielded functional proteins as determined by the ability to complement the defect of *dfi1* null mutants in invasive filamentation (Fig. 3C).

To determine the subcellular localization of Dfi1p, cells containing *DFI1-GFP* were grown on a YPD agar plate, scraped off the plate and observed. The cells showed a marked ring of fluorescence around the cell perimeter and at the cell septum (Fig. 6 panel 3, 5). Identical Dfi1p-GFP localization was seen in cells grown in liquid under conditions that promote growth as either yeast or hyphae (data not shown).

Biochemical fractionation of C-terminally HA-tagged alleles showed that Dfi1p-HA fractionated with the membrane pellet. Following centrifugation of a total extract (Fig. 7A, lane 1) at 300,000g for 1 hr, Dfi1p-HA molecules were recovered from the pellet (Fig. 7A, lane 3) but not the supernatant (Fig. 7A, lane 2). When the pellet fraction was treated with nonionic detergent (Fig. 7A, lanes 4, 5, 8, 9), Dfi1p-HA molecules were extracted. However, treatment with 4 M urea (Fig. 7A, lanes 6 and 7), or 0.1 M $NaCO_3$ (Fig. 7A, lanes 10 and 11) or with buffer only (Fig. 7A, lanes 2 and 3) failed to extract Dfi1p-HA. Therefore, the extractable Dfi1p molecules are integrally associated with a membrane. Based on these combined data, we conclude that Dfi1p is an integral plasma membrane protein.

Dfi1p-HA migrated on SDS-PAGE as a series of isoforms ranging in apparent molecular weight from 170–220kDa (in contrast to the predicted 36kDa). To determine whether O-linked glycosylation contributed to the slow mobility of Dfi1p, tagged *DFI1* alleles were introduced into mutants that were defective in glycosylation (*mnt1/mnt1*, *mnt2/mnt2*, and *mnt1/mnt1 mnt2/mnt2* double null mutants (Munro *et al.*, 2005)). Mnt1p and Mnt2p are required to add the second to fifth mannosyl residues at O-glycosylation sites. In addition, digestion of extracts with peptide:N-glycosidase F (PNGase F; New England Biolabs), an amidase that removes N-linked oligosaccharides from glycoproteins, was used to remove N-linked glycosylation. For these experiments, Dfi1p was tagged with a tandem His6x and HA tag (Dfi1p-TAP), which yielded a functional tagged protein (Fig. 3C). Dfi1p-TAP extracts digested with PNGase F showed a reduction in apparent molecular weight from ~170–220 without digestion (Fig. 7B, lane 5) to ~110kDa after digestion (Fig. 7B, lane 1) indicating that Dfi1p is N-glycosylated.

In the *mnt* null mutants (Fig. 7B, lanes 2–4), the apparent molecular weight of PNGase F-digested Dfi1p-TAP decreased further to approximately 90kDa in the *mnt1/mnt2* double mutant. Increasing enzyme concentration or time of digestion did not increase the mobility of Dfi1p (data not shown). In addition, sequential treatment of Dfi1p-TAP with alpha-mannosidase and PNGase F resulted in a species that migrated at approximately 90kDa, similar in size to the species shown in Fig. 7B, lane 4 (data not shown). Therefore, Mnt1p and Mnt2p are involved in the O-glycosylation of Dfi1p-TAP.

The Dfi1p products obtained by PNGase F and alpha mannosidase digestion or by using glycosylation mutants still exhibited slower mobility than would be expected based on the predicted molecular weight of the Dfi1p polypeptide. Since the first mannose residue in the oligosaccharide remains attached to the polypeptide following alpha mannosidase digestion and *mnt1/mnt2* mutations do not prevent addition of the first residue, this residual carbohydrate could be responsible for the altered mobility of the Dfi1p polypeptide. Therefore, Dfi1p-HA was expressed in an *S. cerevisiae sec53-1* mutant, a mutant blocked in the first step of both N- and O-glycosylation (Ruohola & Ferro-Novick, 1987). Following incubation at the non-permissive temperature (37°C), a Dfi1p-HA species with an apparent molecular weight of 58kDa, rather than the predicted molecular weight of 40kDa for the tagged species, was produced (Fig. 7C). Therefore, we concluded that unglycosylated Dfi1p exhibits an aberrant mobility in SDS-PAGE. In summary, these results show that Dfi1p is a cell surface-associated glycoprotein.

To test for association of Dfi1p with the cell wall, cell wall preparations were isolated from strain pcz11 carrying 2 tagged alleles of *DFI1*. Cell wall fractions were treated with or without Quantazyme, a recombinant beta 1,3-glucanase, to digest the cell wall and release cell wall-bound proteins. The beta-glucanase-treated sample released Dfi1p-TAP molecules (Fig. 7D, lane 2) while the untreated sample did not (Fig. 7D, lane 1). No protein was released upon treatment of isolated cell walls with chitinase or 4M urea (data not shown). Since cell wall-bound Dfi1p was detected via its C-terminal tag, at least some of the wall-bound Dfi1p must retain its transmembrane domain, thus linking the cell wall to the plasma membrane.

In addition, treatment of cell wall preparations from *dfi1/DFI1-TAP*, and *DFI1-TAP/DFI1-TAP* strains with PNGase F, the amidase that removes N-linked oligosaccharides from glycoproteins, released Dfi1p-TAP molecules from the cell wall (Fig. 7D). No Dfi1p-TAP signal was released from *dfi1* null cell walls or cell walls incubated without enzyme. PNGase F may cleave a peptide-carbohydrate bond that links Dfi1p to the cell wall or remove N-linked oligosaccharides that are cross-linked to the wall. We conclude that there is a subpopulation of Dfi1p that is released from cells with detergent and another subpopulation that is released only by enzymatic digestion.

To determine whether the material released by enzymatic digestion constituted a major or minor fraction of the total Dfi1p-TAP, cells were extracted with either detergent treatment alone or with detergent plus PNGase treatment. Following extraction in the presence of detergent, neither Dfi1p-TAP nor the control protein actin were extracted from the insoluble pellet fraction by further treatment with detergent alone (Fig. 7E). However, some Dfip-TAP molecules were extracted from this insoluble fraction by PNGaseF treatment (Fig. 7E). Of the total full length Dfi1-TAP molecules that could be extracted from cells (Fig. 7E lanes labeled T), the majority (about 80–90%) were extracted with detergent alone (Fig. 7E lanes labeled S) and the remainder (10–20%) required PNGase digestion in order to be released from the insoluble fraction (Fig. 7E lanes labeled I).

The $Dfi1_{G273,277L}$p-TAP mutant protein was produced at normal levels and acquired N-linked glycosylation (Fig. 7F). The mutant protein fractionated similarly to WT protein, became crosslinked to the cell wall and localized to the cell perimeter (Fig. 6, panel 7). Therefore, the mutant protein was properly localized but failed to promote Cek1p activation during growth in contact with semi-solid material and failed to adhere to or invade agar medium.

Dfi1p is needed for growth in the presence of cell wall targeting agents

Since some Dfi1p molecules are covalently bound to the cell wall, Dfi1p may play a role in monitoring the status of the cell wall. The *dfi1* null mutant was found to be hypersensitive to the glucan synthase inhibitor Caspofungin (Fig. 8) and the cell wall disturbing agents Congo Red (Fig. 8) and Calcofluor White (data not shown). This hypersensitivity was not remediated by sorbitol. Furthermore, *dfi1* null mutants grew like WT strains in the presence of other stresses (1.5M NaCl, 1M sorbitol, 0.3M $CaCl_2$, 0.1M LiCl, 10mM $MnSO_4$, 0.05% SDS, pH 8.8, 18°C or 42°C; data not shown). These results show that Dfi1p is involved in the maintenance of the cell wall.

In contrast, the $dfi1_{G273,\ 277L}$ mutant strain grew like WT in the presence of Congo Red and Caspofungin (Fig. 8A). Since the mutant protein was properly localized, crosslinked to the wall, and allowed wild type resistance to drugs, the cell wall of the $dfi1_{G273,\ 277L}$ mutant strain did not appear to be compromised. Therefore, the failure of this mutant protein to promote matrix-dependent activation of Cek1p demonstrates that one of the functions of Dfi1p is to participate in activation of signaling in response to growth on a semi-solid surface.

Strain pcz6, (*dfi1/dfi1, CPP1/cpp1*) was as sensitive to Congo Red and Caspofungin as the *dfi1* null mutant (Fig. 8B) despite its ability to invade agar and accumulate matrix-activated Cek1p. Therefore, unlike the defect in invasive filamentation, a reduction in the amount of Cpp1p phosphatase did not bypass the sensitivity of the *dfi1* null mutant to cell wall active drugs. Furthermore, under the conditions of our experiment, strain CCC55 (*cek1* null mutant) grew like WT in the presence of Caspofungin (Fig. 8C). These results show that in addition to its function in promoting Cek1p activation and invasive filamentation, Dfi1p has other functions in cellular physiology.

C. albicans dfi1 null mutant was attenuated in virulence

To determine whether Dfi1p was important for virulence, the behavior of the mutant lacking Dfi1p (pcz25) was analyzed in the hematogenously disseminated murine model, in comparison to a WT strain (pcz24) and the reconstituted null mutant (pcz26). As described in Materials and Methods, 3×10^5 cells from each strain were injected into 16 mice per strain via the lateral tail vein and survival of the mice was monitored as a function of time (Fig. 9). The results showed that both the WT strain and the $DFI1^+$ reconstituted strain killed the majority of the mice while killing by the *dfi1* null mutant was reduced. The survival curves for mice inoculated with WT or *dfi1* null mutant were significantly different ($p<0.001$ by log rank test with Sidak correction for multiple comparisons). Therefore, the strain lacking Dfi1p was attenuated in its ability to cause lethal systemic infection.

DISCUSSION

Numerous species of fungi sense and react to the presence of a surface. For example, the differentiation of *Uromyces appendiculatus* in response to contact with ridges (Hoch *et al.*, 1987, Zhou *et al.*, 1991) and the response of *C. albicans* hyphae to contact with obstructions (Brand *et al.*, 2009, Brand *et al.*, 2007) depend on the rapid activation of mechanosensitive ion channels. In contrast, responses to sustained contact with a matrix, as seen in matrix-induced invasive filamentation of *C. albicans* (Brown *et al.*, 1999), are less well understood. Here we demonstrate that the cell surface protein Dfi1p promotes matrix-dependent Cek1p activation and invasive filamentation of *C. albicans*.

Dfi1p is an integral plasma membrane protein, a fraction of which is covalently bound to the cell wall. Full length Dfi1p can be released from a cell wall fraction by enzymatic digestion of the beta-glucan polymer or by digestion with PNGase F but it is not released by SDS or

chaotropic agents. In addition, the Dfi1p polypeptide is required for normal resistance of *C. albicans* to drugs that perturb the cell wall, such as caspofungin and Congo Red.

Sensing the presence of matrix

The cell wall is a crosslinked mesh of carbohydrate polymers containing proteins that are covalently and noncovalently associated (Chaffin, 2008, Sosinska *et al.*, 2008). Other carbohydrate polymer-rich materials such as mammalian mucus are affected by numerous parameters of the environment, which alter their viscoelastic properties. For example, several factors including the degree of hydration, ion homeostasis, pH, and the presence of oxidizing agents affect lung mucus (Singh & Hollingsworth, 2006). Analogously, contact of a fungal cell wall with a semi-solid surface such as agar may alter the viscoelastic properties of the wall. Seen in this light, the fungal cell wall is not merely a protective structure designed to prevent damage to the cell. Rather, the carbohydrate layers of the cell wall may have an additional important function as sensors of environmental stimuli.

The ability to sense cell wall perturbation underlies the function of other fungal proteins. In *S. cerevisiae*, the plasma membrane proteins Wsc1p and Mid2p have been proposed to be mechanosensors that interact with the cell wall and sense perturbations of the wall (Levin, 2005). A Wsc1p derivative that was engineered to extend through the yeast cell wall was shown to have spring-like properties (Dupres *et al.*, 2009). Importantly, the spring constant of the protein was altered by treatments that perturbed the cell wall, showing that changes in the wall impacted the mechanical properties of the protein (Dupres et al., 2009). Perhaps perturbation of the cell wall affects the behavior of Wsc1p because, like Dfi1p, Wsc1p interacts tightly, sometimes covalently, with the cell wall. Since insoluble fractions are not routinely treated with PNGaseF to detect linked molecules, it may not be unusual for cell surface glycoproteins to be crosslinked to the cell wall.

Sensing of environmental parameters through their effects on the viscoelastic properties of carbohydrates occurs in other cell types. For example, mammalian mucins, highly glycosylated proteins found on the apical surfaces of most simple secretory epithelia (Cullen, 2007), in conjunction with about one hundred other proteins, form mucus, an interconnected mesh of carbohydrate and protein. A membrane tethered mucin, Muc1p, has been proposed to relay changes in pH and ionic composition through their effects on Muc1p conformation. In endothelial cells, the glycocalyx, an extracellular layer composed of polysaccharide-rich molecules, participates in mechanosensing and the response to shear stress (Tarbell & Ebong, 2008). Thus, sensing the effects of the environment on the properties of carbohydrate polymers may be a general mechanism for detecting critical environmental parameters.

MAPK pathways and invasive filamentation

In accord with the model that Dfi1p is needed for the cellular response to changes in the cell wall, the *dfi1* null mutant is hypersensitive to cell wall disturbing agents such as Caspofungin and Congo Red. This hypersensitivity probably reflects a second function for Dfi1p that is not mediated through activation of Cek1p kinase. Although liquid-grown cells challenged with high levels of Congo Red transiently accumulate activated Cek1p (Eisman *et al.*, 2006), reduction in the level of phospho-Cek1p phosphatase Cpp1 did not reduce the hypersensitivity of the *dfi1* mutant strain to Congo Red. Conversely, the $DFI1_{(G273,277L)}$ mutant was resistant to Congo Red despite the fact that this mutant failed to accumulate normal levels of activated Cek1p in response to surface contact. In addition, strain CCC55 (*cek1* null mutant) was not hypersensitive to Caspofungin under the conditions of our experiment, while the *dfi1* null mutant was very sensitive to this agent. These results show

that in addition to its ability to signal, Dfi1p has at least one function that is not mediated through activation of the Cek1p pathway.

Our data suggest that a certain threshold of activated Cek1p kinase is needed to promote invasive filamentation because, in the *dfi1* null mutant, low levels of activated Cek1p were detected in surface-grown cells even though these cells failed to produce invasive filaments. In contrast, the *DFI1* heterozygous strain, which produced slightly reduced levels of contact-activated Cek1p when grown on agar, filamented like the WT strain. Increasing levels of phospho-Cek1p in a *dfi1* null mutant to levels equivalent to the levels in the WT strain by deleting one allele of *CPP1*, resulted in invasive filamentation. Deleting one allele of *CPP1* in a wild type strain increased both invasive filamentation and the levels of phospho-Cek1p but did not result in constitutive filamentation, consistent with the observation that increased levels of phospho-Cek1p were not observed in liquid grown cells lacking Cpp1p (Eisman et al., 2006). All together, these data substantiate the conclusion that there is a critical ratio of phospho-Cek1p to Cek1p that must be achieved in order for a colony to undergo invasive filamentation.

Strain CCC55 (the *cek1* null mutant; (Csank et al., 1998)) was defective in adhering to an agar surface and did not invade the agar. Strain CCC55 also exhibited a delayed embedded filamentation phenotype; however, the phenotype of CCC55 was weaker than the strong filamentation defect observed for pcz5 (the *dfi1* null mutant). The difference in the phenotypes of CCC55 and pcz5 may be related to the fact that CCC55 lacks both phospho-Cek1p and unphosphorylated Cek1p. As seen in *S. cerevisiae* (Cook *et al.*, 1997), unphosphorylated Cek1p may have an inhibitory effect on invasive filamentation.

In addition, residual matrix-dependent Cek1p activation occurs in the *dfi1* null mutant showing that other pathways for matrix-dependent activation of Cek1p exist. Two other plasma membrane proteins that are known to signal to the Cek1p pathway, Sho1p (Roman et al., 2005) and Msb2p (Roman et al., 2009) play roles in the response of cells to growth in contact with matrix. Mutants lacking either of these proteins exhibited delays in filamentation when grown embedded within agar (Table 2). Therefore, the response of cells to matrix involves multiple sensors of cell surface stimuli.

Additionally, the Mkc1p pathway plays a role in regulating invasive filamentation (Kumamoto, 2005). The involvement of multiple MAPKs in regulating invasion is also seen in other fungi such as the phytopathogenic fungus *Magnaporthe grisea*, where the Cek1p ortholog is needed for infection structure formation and the Mkc1p ortholog for penetration (Zhao *et al.*, 2007, Xu & Hamer, 1996, Xu *et al.*, 1998). Thus, like filamentation in liquid medium, invasion during growth on a surface is regulated by multiple pathways.

Matrix sensing and host-Candida interactions

In humans, *C. albicans* is found on the skin, in lungs, in the oral cavity, in the stomach, in the intestines and in the vagina. In healthy individuals, *C. albicans* colonization of these sites is generally benign. If during colonization, the host's immune status was to decline and *C. albicans* organisms were able to adhere and grow on a tissue surface, the fungal responses to the presence of semi-solid material described here would activate invasive filamentation. These events would contribute to the development of invasive lesions and potentially life-threatening candidiasis. The observation that a mutant lacking Dfi1p was attenuated in the ability to produce fatal disseminated infection following intravenous inoculation of mice is consistent with this model. Therefore, the Dfi1p protein, a cell wall component that promotes virulence and invasive filamentation, represents an attractive target for future antifungal drug development.

As a colonizer of human mucosal surfaces, *C. albicans* cells are exposed to many of the same environmental stresses that affect the mucus layers in the host. In both the host and the microorganism, the ability to respond to such stresses with a change in cellular physiology may be mediated through signaling proteins that interact with carbohydrate layers, i.e., Dfi1p for the fungus and mucins for the host. Thus, in this respect, *C. albicans* and its host share the ability to monitor the same environmental parameters. Such fine tuned environmental sensing may be a key element in the success of *C. albicans* as a colonizer and opportunistic pathogen.

EXPERIMENTAL PROCEDURES

Strains

C. albicans strains are listed in Table 2. The *cek1* and *cpp1* null mutants were kindly provided by M. Whiteway and have been described previously (Csank et al., 1997, Csank et al., 1998). The *mnt1* single null, *mnt2* single null and *mnt1, mnt2* double null mutants were the kind gift of Dr. N. Gow (Munro et al., 2005). The *S. cerevisiae sec53* mutant (Ruohola & Ferro-Novick, 1987) was kindly provided by Dr. S. Ferro-Novick. *E. coli* strains DH5α, XL1Blue, or KO1067 (a *dam/dcm* derivative of *E. coli* K12) grown in L broth plus required antibiotic were used to propagate plasmids.

Strain constructions

C. albicans strains were derived from strain BWP17 (Wilson *et al.*, 1999). DNA constructs were introduced into *C. albicans* following the transformation protocol described in (Reuss *et al.*, 2004). Null mutant strains were confirmed by Southern blotting (Ausubel *et al.*, 1989). When needed, strains that carried the *SAT1* (Reuss et al., 2004) flipper cassette were induced to express the site-specific recombinase by growing the cells on liquid YPS for 24 hours. Under these conditions, multiple integrated flipper constructs (*SAT1* and *URA3* (Morschhauser *et al.*, 1999), or *SAT1* and mini*HIS1*, see below) could be resolved at once. Following growth on YPS, colonies that had lost the markers of interest were identified by replica plating onto appropriate media. Deletion of markers from the genome was confirmed by PCR.

After excision of the *URA3* flipper, to restore *URA3* prototrophy, primers pz203 and pz204 were used to PCR amplify the *URA3* locus from the genome of *C. albicans* SC5314. This fragment was cloned into pCR2.1 (Invitrogen), generating pCURA, and sequenced. *ura3/ura3* strains were transformed with a PstI/NcoI *URA3+* fragment purified from pCURA. To construct pcz24 and pcz25, strains were transformed with *HIS1*$^{+}$ and *ARG4*$^{+}$ PCR products and integrations were verified by PCR.

Generation of mutant strains lacking one of 8 candidate genes

For every null mutant shown in Table 1, the following strategy was used: Using SC5314 genomic DNA template, a first round of PCR generated the following two fragments: a 5' fragment, generated with "5'Fragment, Forward Primer" (5' FFP) and 5' Fragment, Reverse Primer (5'FRP), and a 3' fragment, generated with 3'FFP and 3'FRP. Primers are described in Table S1. PCR fragments were gel purified and used as template in a second PCR reaction that included only the 5'FFP and 3'FRP primers to yield a fused fragment that was TOPO cloned into pENTR (Invitrogen). The fused fragments had KpnI and SacI restriction sites at the junction between them. Into this junction, the KpnI/SacI fragments containing the SAT and URA flippers described previously (Morschhauser et al., 1999, Reuss et al., 2004) were cloned. The resulting plasmids were digested with PmeI and BssHII to release the flippers flanked by approximately 500bp of homology on either side of the relevant open reading frame. Gel purified fragments were used for transformations and deletions were

confirmed by Southern Blot. Three independent null mutants were generated for each gene tested.

Construction and use of the URA or SAT placers

These plasmids were engineered from the URA or SAT flippers (Morschhauser et al., 1999, Reuss et al., 2004) (Fig. S1) and allow one step cloning to generate disruption cassettes for gene deletion (described in Supplemental Information).

Construction of tagged alleles of *DFI1*

To complement the *dfi1* null mutation, primers pz03/pz04 were used to generate a PCR fragment encompassing the WT *DFI1* locus. The *DFI1* locus was cloned into vector pNEB193 (New England Biolabs) with SalI and BamHI (pNEBDFI1). After sequencing, the fragment was cloned into the URAplacer (pUPL) generating pUPLDFI1. To cross the gene back into the chromosome, pUPLDFI1 was cut with BglII, which cuts at a unique site within the 5' UTR of *DFI1* and the linearized DNA was transformed into a *dfi1* null strain.

C-terminally tagged *DFI1* alleles were constructed as follows: using primers pz175/176, BspEI and BclI sites were introduced after the last codon in *DFI1* to generate pNEBDFI1-Ctags. The HA and GFP tags were amplified using primers pz179/180 (HA_3), and pz195/196 (GFP) and plasmids pFA6AHA3 (Longtine *et al.*, 1998) and pYEGFP (Cormack *et al.*, 1997) as templates and introduced into the BspEI/BclI sites of pNEBDFI1-Ctags. All tags were linked to *DFI1* via a Ser-Gly_4 amino acid linker and all constructs retained the native 3' UTR of the *DFI1* locus.

Mutant alleles of *DFI1* were generated by overlap PCR. Briefly, using pNEBDFI1-HA (or TAP or GFP) as template, two mutagenic primers were used to generate two overlapping fragments that were fused together in a final round of PCR. The first round primer pairs used to generate the $G_{273,\ 277}L$ mutated allele were pz247/pz63 and pz134/pz248. The second round PCR used primer pair pz134/pz63. This fragment was digested with XhoI and MluI and moved into pSPLDFI1-TAP (*DFI1-TAP* in the SAT placer) or pSPLDFI1-GFP (*DFI1-GFP* in the SAT placer) to generate pSPLDFI1$_{G273,277L}$-TAP or pSPLDFI1$_{G273,277L}$-GFP plasmids. To generate pSPLDFI1-TAP (*DFI1-TAP* in the SAT placer) an oligomer encoding GSGGGHHHHHH was cloned in frame at the BspEI site immediately upstream of the HA tag present in pSPLDFI1-HA.

For introduction of TAP-tagged DFI1 alleles into *mnt1* null, *mnt2* null and *mnt1mnt2* double null backgrounds, a portion of the 3' UTR from the *DFI1* locus (flanked by BssHII and BglII sites) was amplified by PCR and cloned upstream of the 5' UTR and the rest of the *DFI1* locus in pSPLDFI1-TAP (*DFI1-TAP* in the SAT placer) so that a unique BglII site was present between the 3' and 5' UTR fragments. The resulting plasmid, pSPL2XO-DFI-TAP, was linearized with BglII prior to transformation.

For expression of *DFI1-HA* in *S. cerevisiae*, the tagged *DFI1* locus was amplified with primers pz22 and pz201 and cloned onto pYES2.1/V5-His-TOPO (Invitrogen).

Molecular biology methods

Unless otherwise stated, all PCR reactions used Hi-Fi polymerase (Invitrogen), Pfu Turbo or Ultra (Stratagene) following the manufacturers' recommendations. Primers are listed in Tables S1 and S2. All restriction enzymes and DNA ligase were purchased from New England Biolabs. All constructs generated by PCR were confirmed by sequencing. Protein sequence analysis was performed with the Biology WorkBench, http://workbench.sdsc.edu, or with BLAST.

Growth of cells

C. albicans was routinely grown in YPD (1% Yeast extract, 2% Peptone, 2% glucose), YPS (1% Yeast extract, 2% Peptone, 2% sucrose) or CM (complete medium minus amino acid or uridine; (Ausubel et al., 1989)) at 25°C, 30°C or 37°C. Nourseothricin (Werner BioAgents, Germany) was used at 200μg/ml.

Embedded filamentation assays

The growth of colonies under embedded conditions was performed as described previously (Brown et al., 1999) with the following modifications: in order to minimize plate to plate variations in water activities, following 4 hours of growth of a diluted overnight culture, 25ml of lukewarm 1% YPS agar was pipetted on top of a drop of medium containing approximately 150 cells. Solidified plates were placed inside a humidified chamber for the duration of the experiment in order to minimize uneven drying of the plates. The plates were incubated at either 25°C or 37°C. Filamentous colonies were defined as colonies with at least 20 visible filaments. Each assay described was repeated at least three times.

Agar invasion assays

Cells were grown on the surface of YPSA medium for 4 days at 25°C. Colonies were washed off the surface of the agar with a stream of water and gentle rubbing. Adherent cells remaining on the surface of the agar were photographed at 4× power. A sliver of agar was cut through the center of a colony, flipped on its side and photographed in cross section at 4× power.

Liquid filamentation

Cultures were grown in liquid YPD supplemented with 10% bovine serum, RPMI medium, or Spider medium at 37°C. At various times, cells were collected, stained with 10 μg/ml Calcofluor White (Sigma), washed and photographed. These assays were performed in triplicate.

Caspofungin or Congo Red treatment

To test for sensitivity to cell wall disturbing agents, cultures grown at 30°C were diluted into fresh YPD and allowed to grow at 30°C to OD_{600}~1.5. Serial dilutions were spotted on YPD plates or YPD plates supplemented with either 200 μg/ml Congo Red or 90ng/ml Caspofungin. For Fig. 8C, plates were supplemented with 150ng/ml Capsofungin or 250 μg/ml Congo Red. Plates were incubated at 30°C for times indicated. Sensitivity was tested at least three times.

Protein extraction and immunoblotting

To characterize the levels of activated MAPK, cells were grown and extracted as described (Kumamoto, 2005). Cells were grown in liquid medium for 72 hours or on the surface of agar medium for 72 or 96 hrs. Extracts were fractionated by SDS-PAGE and analyzed by immunoblotting. For Western Blotting, 80–100 μg total protein (for phospho-MAPK or Cek1p) or 5–20 μg (for actin) was loaded per sample and separated on an 8% SDS-PAGE (MAPK or Cek1p) or 12% (actin). After transfer onto PVDF membranes (Millipore, 0.2 μm) by standard protocols, blots were initially probed with anti-dually phosphorylated p42/p44 MAPK rabbit polyclonal antiserum (Cell Signalling; CS-9101) at 1:1000 (Figs. 1 and 4), overnight at 4°C, or with anti-dually phosphorylated p42/p44 MAPK rabbit monoclonal antibody (Cell Signalling; CS-4370) (Fig. 5). Goat-anti-rabbit IgG-HRP (Zymed, 62-6120) was used at 1:5000 dilution as secondary antibody. Following detection using Amersham ECL system, the blot was stripped and reprobed with anti-Cek1 rabbit polyclonal (1:3000; kindly provided by Dr J. Pl·) overnight at 4°C. Anti-Cek1 signal was detected using NEN

luminol. Actin loading controls were probed at 1:15000 with rabbit anti-actin (Sigma, A5060) and detected as described above. ImageQuant software was used to quantify results. Comparisons of MAPK activation in WT and *dfi1* null mutants were performed more than 10 times and results were consistent.

To detect Dfi1p-HA, anti-HA (1:1000 overnight incubation at 4°C) obtained from the Tufts University Monoclonal Grasp Center was used. Goat-anti-mouse-HRP (Cell Signaling, 7076) was used as secondary and the signal was detected with NEN luminol.

Subcellular fractionation

Cells were grown in liquid or on plates and collected over ice as described above. All subsequent steps were performed at 4°C. Following a PBS wash, cell pellets were resuspended in 1× EB buffer (0.3M Sorbitol, 10mM Tris pH 7.5, 100mM NaCl, 1mM $MgSO_4$, 1mM EDTA) plus protease inhibitors (1mM PMSF and Fungal Specific protease inhibitor (Sigma P8215) at 1ml/20g cell paste) and broken using a French Press (18000psi) or a bead beater (following standard protocols). The extracts were spun twice at 500×g to remove unbroken cells and the supernatant was centrifuged at 300,000×g for 1 hour in a Beckman ultracentrifuge (TLA100.3 rotor). The 300K supernatant was removed and the remaining membrane pellet was homogenized by hand with a Teflon-glass homogenizer in the same buffer (EB), or EB supplemented with 1% TritonX-100, EB+4M urea, EB+4M urea+1% TritonX-100, or EB+0.1M Na_2CO_3. Samples were incubated on ice for 30 minutes, and then centrifuged at 13,000×g for 30 minutes in a tabletop centrifuge at 4°C. The supernatant was removed and the pellet was resuspended in a volume equal to the original volume of EB+1%SDS. Equal volumes of samples were loaded onto 6% SDS-PAGE gels. Fractionation experiments were repeated numerous times.

Cell wall extraction, glucanase, chitinase, and PNGase F digestion

Cells were grown in YPD at 30°C to an OD_{600} of 1.0, and the cell wall fraction was isolated, digested and analyzed as described (Pitarch *et al.*, 2008). For digestion, 170 mg wet weight of the cell wall fraction was incubated with 1500U/gm wet weight of Quantazyme (MP) at 37°C for 4–16 hours. Alternatively, 200 mg wet weight of cell wall was incubated with 20,000 units of PNGase F (New England Biolabs P0704) at 37°C for 4 hours. Samples incubated with or without enzyme were centrifuged at 500×g for 10 min. at 4°C, and the supernatants were precipitated with TCA and analyzed by Western Blotting with anti-HA antibody. Experiments were repeated three times.

For PNGaseF digestion of whole cells, cells were grown in YPD at 30°C to an $OD600_{nm}$ of approximately 1.5. Cells were harvested by centrifugation and cell pellets were washed three times with ice cold water. The cell pellets (~50 mg) were resuspended in 600 µl 1 × RIPA buffer (50 mM Tris HCl pH 8.0, 150 mM NaCl, 0.1% SDS, 1% NP40, 0.5% sodium deoxycholate) plus protease inhibitors (2 mM PMSF, Roche Complete Protease Inhibitor cocktail and Sigma Inhibitor cocktail). After addition of 1/10 volume Denaturation buffer (5% SDS, 400 mM DTT), samples were boiled for 10 minutes and then frozen in a dry ice/ethanol bath, followed by thawing in an ice/water bath. This process was repeated two more times and then samples were boiled for a final time. Following this fourth boiling, 1/10 volume each of Buffer G7 (0.5M $NaPO_4$ pH 7.5) and NP40 (10% stock) and 5 µl of Sigma Inhibitor Cocktail mix were added. Half of this sample was transferred to a new tube ("Total") and the other half was centrifuged at 15,000×g in a refrigerated table top microcentrifuge at 4°C for 15 minutes. The supernatant was transferred to a new tube ("Soluble"). The remaining pellet was washed three times with ice cold water, and collected by centrifugation at 200×g (4°C) for one minute. The washed pellets were resuspended in the same volume as the Soluble samples, in the same buffer, and labeled "Insoluble". An

aliquot from each fraction (Total, Soluble and Insoluble) was transferred to a new tube to serve as an undigested control. The remainder of each sample received PNGase F (500U/ µl, NEB). Samples with and without PNGaseF were incubated at 37°C for 3.5 hours. Following digestion, the tubes were centrifuged at 200×g for 15 sec. and the supernatants were transferred to new tubes and processed for SDS-PAGE on a 7.5% gel, followed by immunoblotting with anti-HA or anti-actin antibody. A second gel, carrying the same samples, was run, transferred to PVDF and stained with Coomassie Blue; discreet bands were detected in all lanes, and no evidence of protein degradation was observed in any of the lanes.

Intravenously inoculated mouse model of candidiasis

C. albicans strains were grown overnight in CM-Ura medium and harvested by centrifugation at 3,250×g. Cells were washed with PBS twice, resuspended, counted and adjusted to a density of 3×10^6 cells ml^{-1} in PBS. The cell suspension (0.1 ml containing 3×10^5 cells) was injected into the lateral tail vein of female CF1 mice (18 to 20g; Charles River Laboratories, Wilmington, MA). Each *C. albicans* strain was tested in two experiments in a total of 16 mice and combined results of the two experiments are shown. Survival of mice was monitored daily after infection with *C. albicans* for 21 days. Statistical analysis of survival curves using the log rank test was performed with SAS version 9.2.

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REFERENCES

Ahn IP, Kim S, Choi WB, Lee YH. Calcium restores prepenetration morphogenesis abolished by polyamines in *Colletotrichum gloeosporioides* infecting red pepper. FEMS Microbiol Lett. 2003; 227:237–241. [PubMed: 14592714]

Ausubel, F.; Brent, R.; Kingston, R.; Moore, D.; Seidman, J.; Smith, J.; Struhl, K. Current Protocols in Molecular Biology. New York, NY: J. Wiley & Sons, Inc.; 1989.

Berman J. Morphogenesis and cell cycle progression in *Candida albicans*. Curr Opin Microbiol. 2006; 9:595–601. [PubMed: 17055773]

Bershadsky AD, Balaban NQ, Geiger B. Adhesion-dependent cell mechanosensitivity. Annu Rev Cell Dev Biol. 2003; 19:677–695. [PubMed: 14570586]

Brand A, Lee K, Veses V, Gow NA. Calcium homeostasis is required for contact-dependent helical and sinusoidal tip growth in *Candida albicans* hyphae. Mol Microbiol. 2009

Brand A, Shanks S, Duncan VM, Yang M, Mackenzie K, Gow NA. Hyphal orientation of *Candida albicans* is regulated by a calcium-dependent mechanism. Curr Biol. 2007; 17:347–352. [PubMed: 17275302]

Brosig B, Langosch D. The dimerization motif of the glycophorin A transmembrane segment in membranes: importance of glycine residues. Protein Sci. 1998; 7:1052–1056. [PubMed: 9568912]

Brown DH Jr, Giusani AD, Chen X, Kumamoto CA. Filamentous growth of *Candida albicans* in response to physical environmental cues and its regulation by the unique *CZF1* gene. Mol Microbiol. 1999; 34:651–662. [PubMed: 10564506]

Chaffin WL. *Candida albicans* cell wall proteins. Microbiol Mol Biol Rev. 2008; 72:495–544. [PubMed: 18772287]

Cook JG, Bardwell L, Thorner J. Inhibitory and activating functions for MAPK Kss1 in the S. cerevisiae filamentous-growth signalling pathway. Nature. 1997; 390:85–88. [PubMed: 9363895]

Cormack BP, Bertram G, Egerton M, Gow NA, Falkow S, Brown AJ. Yeast-enhanced green fluorescent protein (yEGFP)a reporter of gene expression in *Candida albicans*. Microbiology. 1997; 143(Pt 2):303–311. [PubMed: 9043107]

Csank C, Makris C, Meloche S, Schroppel K, Rollinghoff M, Dignard D, Thomas DY, Whiteway M. Derepressed hyphal growth and reduced virulence in a VH1 family-related protein phosphatase mutant of the human pathogen *Candida albicans*. Mol Biol Cell. 1997; 8:2539–2551. [PubMed: 9398674]

Csank C, Schroppel K, Leberer E, Harcus D, Mohamed O, Meloche S, Thomas DY, Whiteway M. Roles of the Candida albicans mitogen-activated protein kinase homolog, Cek1p, in hyphal development and systemic candidiasis. Infect Immun. 1998; 66:2713–2721. [PubMed: 9596738]

Cullen PJ. Signaling mucins: the new kids on the MAPK block. Crit Rev Eukaryot Gene Expr. 2007; 17:241–257. [PubMed: 17725491]

Curran AR, Engelman DM. Sequence motifs, polar interactions and conformational changes in helical membrane proteins. Curr Opin Struct Biol. 2003; 13:412–417. [PubMed: 12948770]

Doehlemann G, Berndt P, Hahn M. Different signalling pathways involving a Galpha protein, cAMP and a MAP kinase control germination of *Botrytis cinerea* conidia. Mol Microbiol. 2006; 59:821–835. [PubMed: 16420354]

Dupres V, Alsteens D, Wilk S, Hansen B, Heinisch JJ, Dufrene YF. The yeast Wsc1 cell surface sensor behaves like a nanospring in vivo. Nat Chem Biol. 2009; 5:857–862. [PubMed: 19767735]

Eisman B, Alonso-Monge R, Roman E, Arana D, Nombela C, Pla J. The Cek1 and Hog1 mitogen-activated protein kinases play complementary roles in cell wall biogenesis and chlamydospore formation in the fungal pathogen *Candida albicans*. Eukaryot Cell. 2006; 5:347–358. [PubMed: 16467475]

Gancedo JM. Control of pseudohyphae formation in *Saccharomyces cerevisiae*. FEMS Microbiol Rev. 2001; 25:107–123. [PubMed: 11152942]

Gimeno CJ, Ljungdahl PO, Styles CA, Fink GR. Unipolar cell divisions in the yeast *S. cerevisiae* lead to filamentous growth: regulation by starvation and RAS. Cell. 1992; 68:1077–1090. [PubMed: 1547504]

Gow NA. Germ tube growth of *Candida albicans*. Curr Top Med Mycol. 1997; 8:43–55. [PubMed: 9504066]

Hoch HC, Staples RC, Whitehead B, Comeau J, Wolf ED. Signaling for Growth Orientation and Cell Differentiation by Surface Topography in *Uromyces*. Science. 1987; 235:1659–1662. [PubMed: 17795599]

Ingber DE. Mechanical control of tissue morphogenesis during embryological development. Int J Dev Biol. 2006; 50:255–266. [PubMed: 16479493]

Kumamoto CA. A contact-activated kinase signals *Candida albicans* invasive growth and biofilm development. Proc Natl Acad Sci U S A. 2005; 102:5576–5581. [PubMed: 15800048]

Kumamoto CA, Vinces MD. Alternative *Candida albicans* lifestyles: growth on surfaces. Annu Rev Microbiol. 2005; 59:113–133. [PubMed: 16153165]

Kuo K, Hoch HC. Germination of *Phyllosticta ampelicida* Pycnidiospores: Prerequisite of Adhesion to the Substratum and the Relationship of Substratum Wettability. Fungal Genet Biol. 1996; 20:18–29. [PubMed: 8812283]

Levin DE. Cell wall integrity signaling in *Saccharomyces cerevisiae*. Microbiol Mol Biol Rev. 2005; 69:262–291. [PubMed: 15944456]

Longtine MS, McKenzie A 3rd, Demarini DJ, Shah NG, Wach A, Brachat A, Philippsen P, Pringle JR. Additional modules for versatile and economical PCR-based gene deletion and modification in *Saccharomyces cerevisiae*. Yeast. 1998; 14:953–961. [PubMed: 9717241]

Migliaccio F, Piconese S. Spiralizations and tropisms in *Arabidopsis* roots. Trends Plant Sci. 2001; 6:561–565. [PubMed: 11738380]

Morschhauser J, Michel S, Staib P. Sequential gene disruption in *Candida albicans* by FLP-mediated site-specific recombination. Mol Microbiol. 1999; 32:547–556. [PubMed: 10320577]

Munro CA, Bates S, Buurman ET, Hughes HB, Maccallum DM, Bertram G, Atrih A, Ferguson MA, Bain JM, Brand A, Hamilton S, Westwater C, Thomson LM, Brown AJ, Odds FC, Gow NA. Mnt1p and Mnt2p of *Candida albicans* are partially redundant alpha-1,2-mannosyltransferases

that participate in O-linked mannosylation and are required for adhesion and virulence. J Biol Chem. 2005; 280:1051–1060. [PubMed: 15519997]

Odds FC. *Candida* infections: an overview. Crit Rev Microbiol. 1987; 15:1–5. [PubMed: 3319417]

Pitarch A, Nombela C, Gil C. Cell wall fractionation for yeast and fungal proteomics. Methods Mol Biol. 2008; 425:217–239. [PubMed: 18369900]

Reuss O, Vik A, Kolter R, Morschhauser J. The *SAT1* flipper, an optimized tool for gene disruption in *Candida albicans*. Gene. 2004; 341:119–127. [PubMed: 15474295]

Roman E, Cottier F, Ernst JF, Pla J. Msb2 signaling mucin controls activation of Cek1 mitogen-activated protein kinase in *Candida albicans*. Eukaryot Cell. 2009; 8:1235–1249. [PubMed: 19542310]

Roman E, Nombela C, Pla J. The Sho1 adaptor protein links oxidative stress to morphogenesis and cell wall biosynthesis in the fungal pathogen *Candida albicans*. Mol Cell Biol. 2005; 25:10611–10627. [PubMed: 16287872]

Ruohola H, Ferro-Novick S. Sec53, a protein required for an early step in secretory protein processing and transport in yeast, interacts with the cytoplasmic surface of the endoplasmic reticulum. Proc Natl Acad Sci U S A. 1987; 84:8468–8472. [PubMed: 3317409]

Singh PK, Hollingsworth MA. Cell surface-associated mucins in signal transduction. Trends Cell Biol. 2006; 16:467–476. [PubMed: 16904320]

Smith SO, Song D, Shekar S, Groesbeek M, Ziliox M, Aimoto S. Structure of the transmembrane dimer interface of glycophorin A in membrane bilayers. Biochemistry. 2001; 40:6553–6558. [PubMed: 11380249]

Soll DR, Galask R, Schmid J, Hanna C, Mac K, Morrow B. Genetic dissimilarity of commensal strains of *Candida* spp. carried in different anatomical locations of the same healthy women. J Clin Microbiol. 1991; 29:1702–1710. [PubMed: 1761692]

Sosinska GJ, de Groot PW, Teixeira de Mattos MJ, Dekker HL, de Koster CG, Hellingwerf KJ, Klis FM. Hypoxic conditions and iron restriction affect the cell-wall proteome of *Candida albicans* grown under vagina-simulative conditions. Microbiology. 2008; 154:510–520. [PubMed: 18227255]

Sudbery P, Gow N, Berman J. The distinct morphogenic states of *Candida albicans*. Trends Microbiol. 2004; 12:317–324. [PubMed: 15223059]

Tarbell JM, Ebong EE. The endothelial glycocalyx: a mechano-sensor and -transducer. Sci Signal. 2008; 1(pt8)

Tucker SL, Talbot NJ. Surface attachment and pre-penetration stage development by plant pathogenic fungi. Annu Rev Phytopathol. 2001; 39:385–417. [PubMed: 11701871]

Wilson RB, Davis D, Mitchell AP. Rapid hypothesis testing with *Candida albicans* through gene disruption with short homology regions. J Bacteriol. 1999; 181:1868–1874. [PubMed: 10074081]

Xu JR, Hamer JE. MAP kinase and cAMP signaling regulate infection structure formation and pathogenic growth in the rice blast fungus *Magnaporthe grisea*. Genes Dev. 1996; 10:2696–2706. [PubMed: 8946911]

Xu JR, Staiger CJ, Hamer JE. Inactivation of the mitogen-activated protein kinase Mps1 from the rice blast fungus prevents penetration of host cells but allows activation of plant defense responses. Proc Natl Acad Sci U S A. 1998; 95:12713–12718. [PubMed: 9770551]

Zhao X, Mehrabi R, Xu JR. Mitogen-activated protein kinase pathways and fungal pathogenesis. Eukaryot Cell. 2007; 6:1701–1714. [PubMed: 17715363]

Zhou XL, Stumpf MA, Hoch HC, Kung C. A mechanosensitive channel in whole cells and in membrane patches of the fungus *Uromyces*. Science. 1991; 253:1415–1417. [PubMed: 1716786]

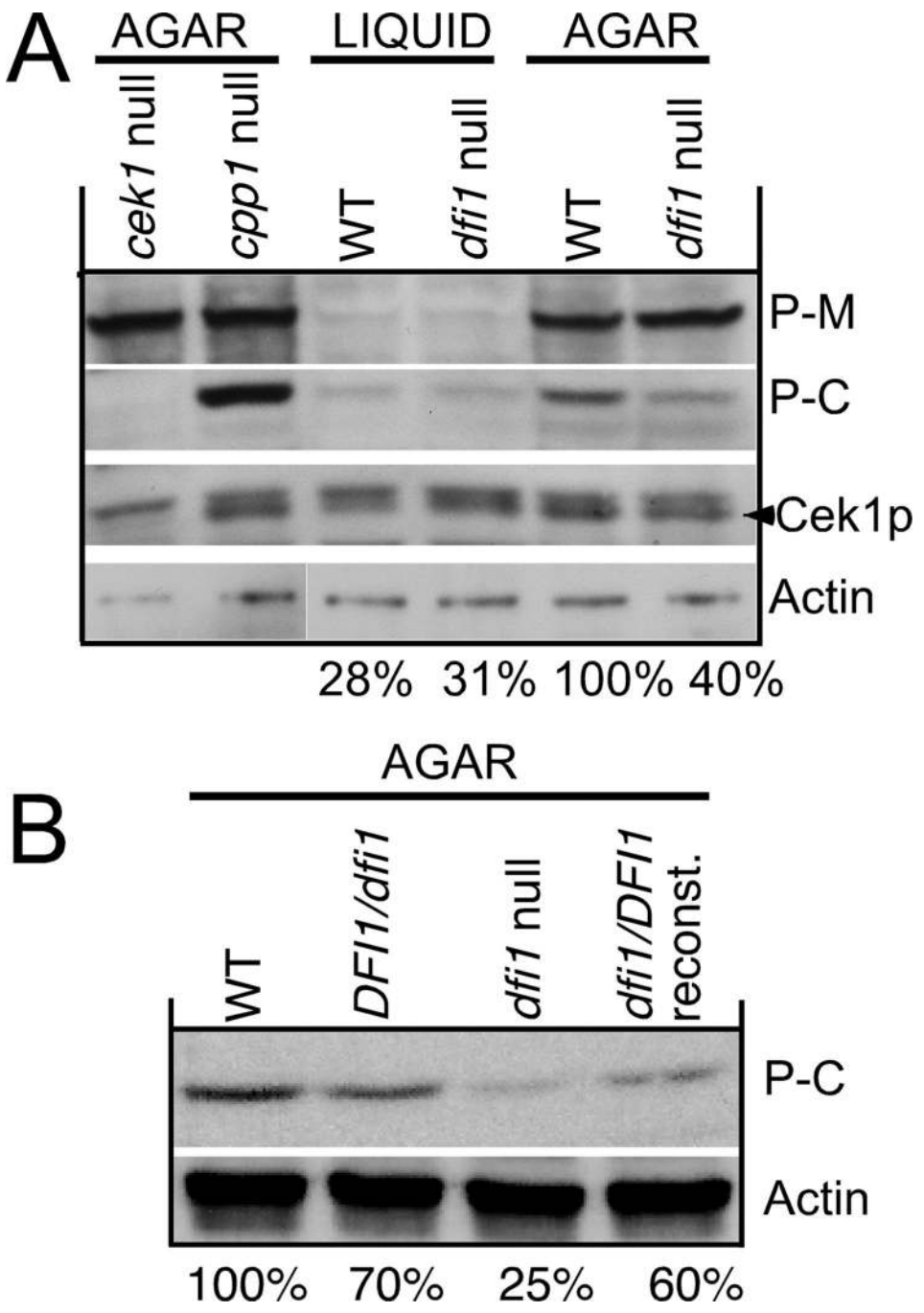

Fig. 1. Matrix-dependent activation of Cek1p is partially Dfi1p dependent

A. Strains were grown on YPS agar (surface-grown cells) or in liquid medium at 25°C for 3 days, harvested and extracted as described in Experimental procedures. One hundred μg of total protein per lane was analyzed by SDS-PAGE and immunoblotting. Top two panels correspond to signal detected with anti-phospho-p42-44 MAP Kinase polyclonal antiserum. The third panel corresponds to the same blot stripped and reprobed with anti-Cek1p polyclonal. Bottom-most panel corresponds to a different gel carrying 5 μg protein extract per lane probed with anti-actin polyclonal. Lanes (left to right): 1, *cek1* null (CCC55, surface-grown); 2, *cpp1* null (CCC81, surface-grown); 3, WT (pcz1, liquid); 4, *dfi1* null (pcz5, liquid); 5, WT (pcz1, surface-grown); 6, *dfi1* null (pcz5, surface-grown). Percentages

indicate the relative amounts of dually-phosphorylated Cek1p signal, normalized to actin levels. P-M, phospho-Mkc1p; P-C, phospho-Cek1p; black arrowhead indicates Cek1p. B. Strains were grown on YPS agar at 25°C for 4 days, harvested and extracted as in Experimental procedures. Eighty μg of total protein per lane was analyzed by SDS-PAGE and immunoblotting. Top panel, anti-phospho-p42-44 MAP Kinase polyclonal antiserum. Bottom panel, a different gel carrying 20 μg/lane probed with anti-actin polyclonal. Lanes (left to right): 1, WT (pcz1); 2, *DFI1/dfi1* heterozygote (pcz2); 3, *dfi1* null (pcz5); 4, *dfi1/DFI1* reconstituted null mutant (pcz7). Percentages indicate the relative amounts of dually-phosphorylated Cek1p signal, normalized to actin levels. P-C, phospho-Cek1p.

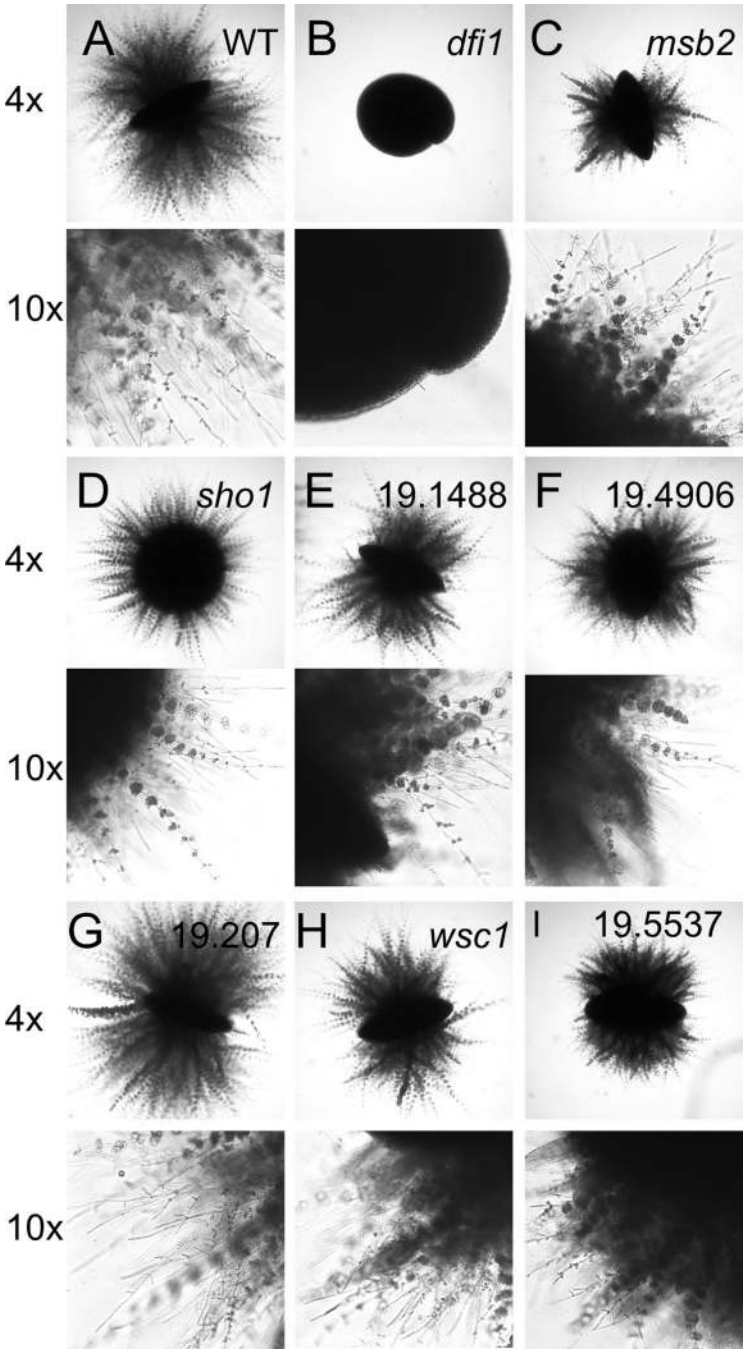

Fig. 2. Filamentation during growth under embedded conditions

Cells from strains listed in Table 1 were grown embedded in YPS+uridine 1% agar for 4 days at 25°C. Representative colonies were photographed at 4× (above) and 10× (below). Strains were: A, pcz1 (WT); B, pcz5 (*dfi1* null mutant); C, *msb2* null mutant; D, *sho1* null mutant; E, *ORF19.1488* null mutant; F, *ORF19.4906* null mutant; G, *ORF19.207* null mutant; H, *wsc1* null mutant; I, *ORF19.5537* null mutant.

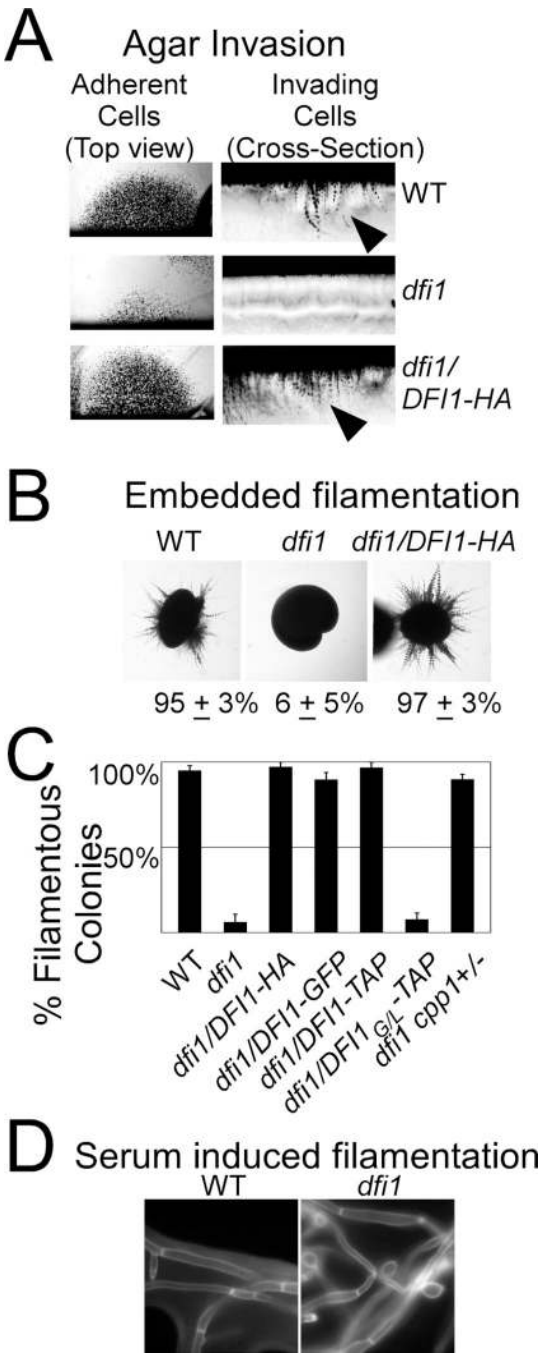

Fig. 3. *dfi1* null mutant is defective in agar invasion and embedded filamentation

A. Cells of strains WT (pcz1), *dfi1* null (pcz5), and *dfi1/DFI1-HA* reconstituted mutant (pcz8) were incubated for 4 days at 25°C on the surface of YPSA medium. Colonies were washed off the surface of the agar. Adherent cells remaining on the surface of the agar were photographed at 4× power (left column). The agar was cut through the center of the colony and photographed in cross section at 4× power (right column). Arrowhead indicates invading filaments.

B. Cells of strains WT (pcz1), *dfi1* null (pcz5), and *dfi1/DFI1-HA* reconstituted mutant (pcz8) were incubated for 4 days at 25°C embedded in YPS agar. Filamentous colonies were scored as described in Experimental procedures.

C. Strains carrying the indicated constructs were embedded in YPS agar and incubated for up to 5 days at 25°C. Filamentous colonies were scored as above. The average of at least three experiments is shown. Error bars indicate standard deviation.
D. Cells of strains WT (pcz1) and *dfi1* null (pcz5) were grown in YPD supplemented with 10% bovine serum at 37°C for 2 hours. Cells were stained with Calcofluor White to visualize the cell wall. (Magnification 60X.)

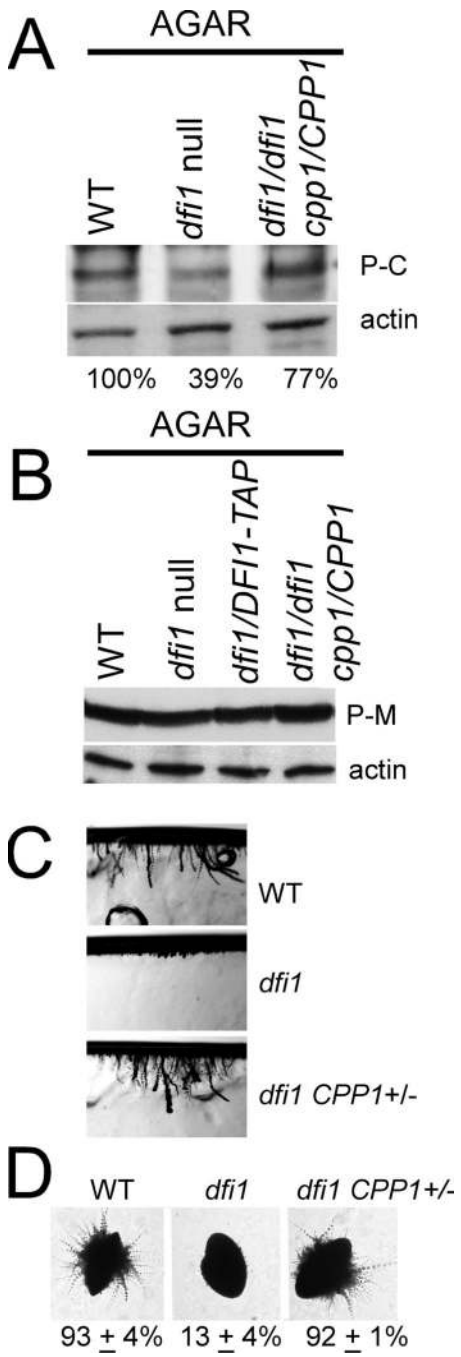

Fig. 4. Correlation between matrix-dependent activation of Cek1p and invasive filamentation

A, and B. Cells were grown on the surface of YPS agar at 25°C for 4 days, extracted and processed as in Fig. 1. Upper blots, 100 μg total protein/lane probed with anti-phospho MAPK polyclonal (A) or monoclonal (B). Lower blots, 5 μg total protein/lane probed with anti-actin polyclonal. Lanes in panel A (left to right): 1, WT (pcz1); 2, *dfi1* null (pcz5); 3, *dfi1/dfi1*, *cpp1/CPP1* (pcz6). Percentages show relative amounts of dually-phosphorylated Cek1p signal, normalized to actin levels. Lanes in panel B (left to right): 1, WT (pcz1); 2, *dfi1* null (pcz5); 3, *dif1/DFI1-TAP*; 4, *dfi1/dfi1*, *cpp1/CPP1* (pcz6). P-C, phospho-Cek1p; P-M, phospho-Mkc1p.

C. Cells of strains WT (pcz1), *dfi1* null (pcz5), and *dfi1/dfi1*, *cpp1/CPP1* (pcz6) were incubated for 4 days at 25°C on the surface of YPSA medium. Non-adherent cells were removed by washing and the agar beneath the colony was imaged in cross section as described in Fig. 3A.

D. Cells of strains WT (pcz1); *dfi1* null (pcz5), and *dfi1/dfi1*, *cpp1/CPP1* (pcz6) were embedded in YPS agar and incubated for 4 days at 25°C. Numbers show percent filamentous colonies, scored as described.

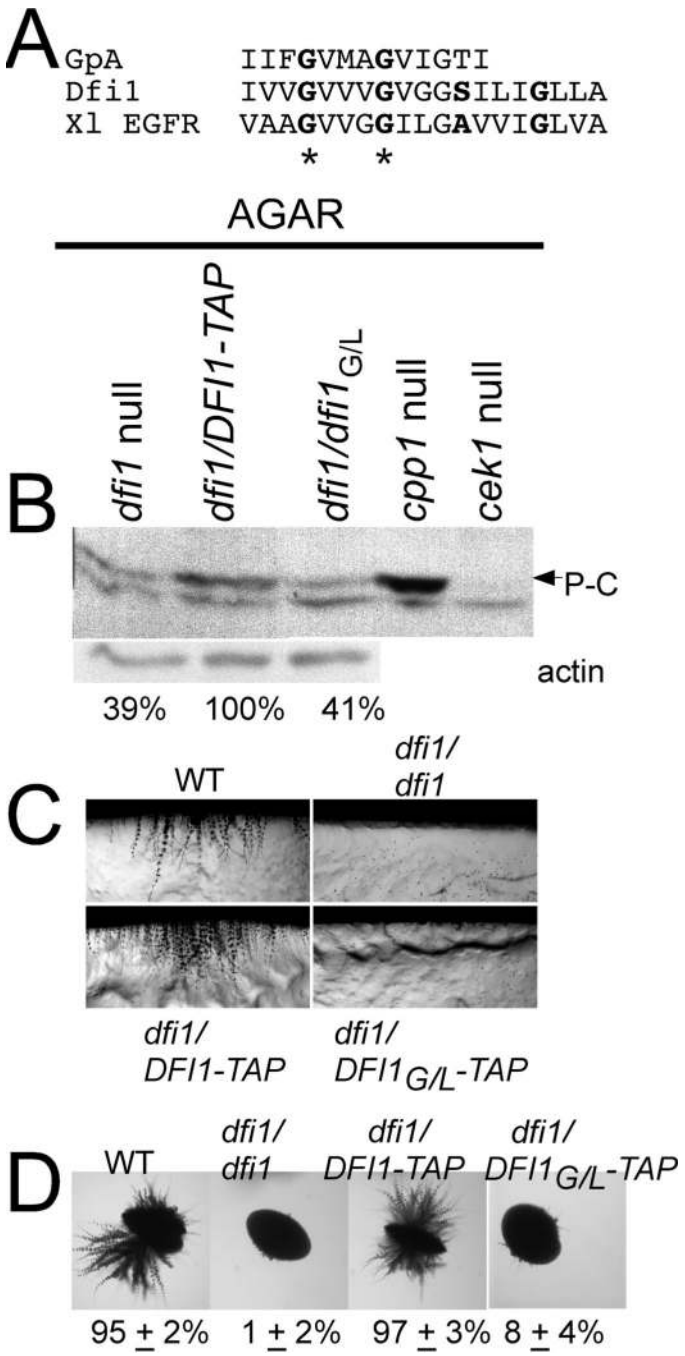

Fig. 5. A GxxxG motif found in the transmembrane segment of Dfi1p is needed to signal to Cek1p kinase

A. The GxxxG motif of Dfi1p, Glycophorin A (GpA), and *Xenopus laevis* Epidermal Growth Factor Receptor. * symbols indicate the residues changed to Leu in the *dfi1*$_{G273,\ 277L}$ mutant.

B. Cells of strains *dfi1* null (pcz5), *dfi1/DFI1-TAP* (pcz9), and *dfi1/dfi1*$_{G273,\ 277L}$*-TAP* (pcz22) were grown on YPS agar plates at 25°C for 4 days, extracted and processed for immunoblotting as described Fig. 1A. Top immunoblot corresponds to 100 µg total protein/lane probed with anti-phospho-p42-p44 MAP kinase monoclonal. Bottom immunoblot corresponds to 5 µg total protein/lane run on a separate gel and probed with anti-actin

polyclonal. The percentages indicate the relative amount of dually-phosphorylated Cek1p signal, normalized to actin levels. The arrowhead indicates the position of dually-phosphorylated Cek1p.

C. Representative images of invading filaments obtained after growing strains WT (pcz1), *dfi1/dfi1* null (pcz5), *dfi1/DFI1-TAP* (pcz9), and *dfi1/dfi1*$_{G273,\ 277L}$*-TAP* (pcz22) on the surface of YPSA plates for 4 days at 25°C. Non-adherent cells were removed by washing and the agar beneath the colony was imaged in cross section as described in Fig. 3A.

D. Representative embedded colonies of WT (pcz1), *dfi1/dfi1* null (pcz5), *dfi1/DFI1-TAP* (pcz9), and *dfi1/dfi1*$_{G273,\ 277L}$*-TAP* (pcz22) strains. Cells were grown as described in Fig. 3B. Filamentous colonies were scored as described in Experimental procedures.

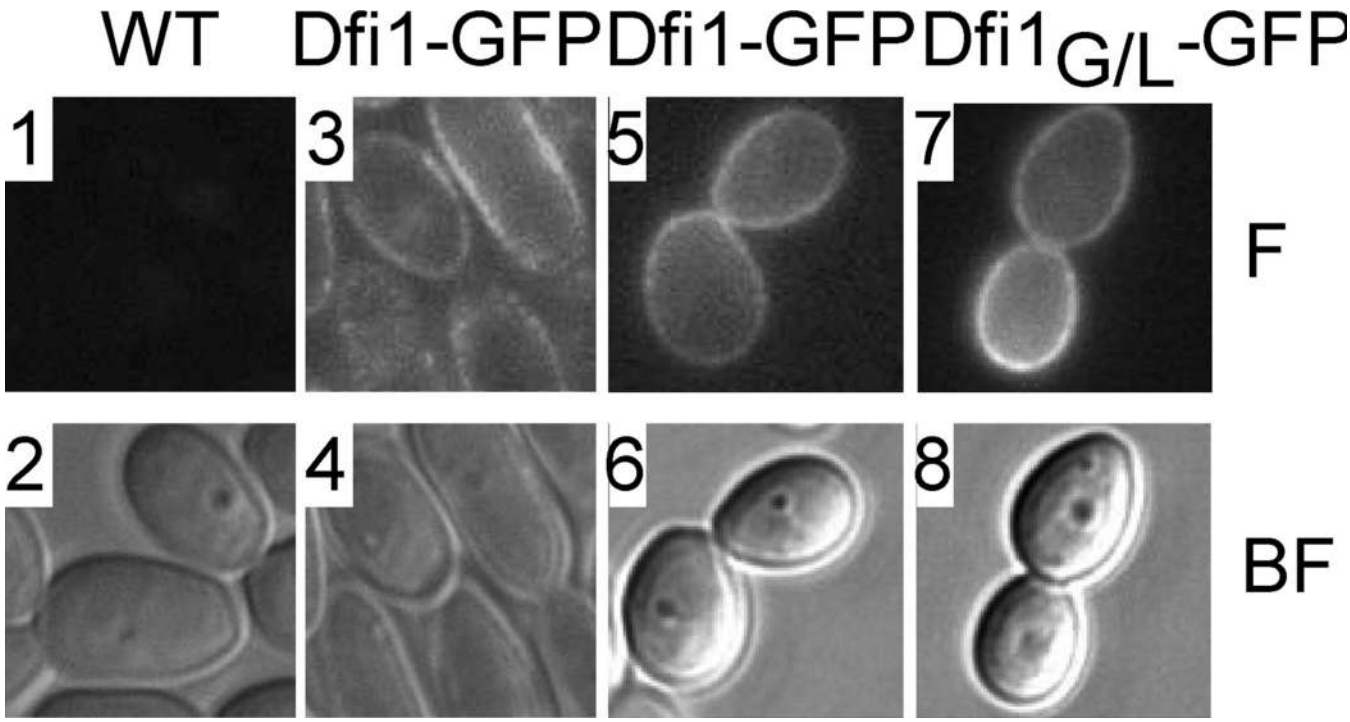

Fig. 6. Dfi1p-GFP localized to the cell periphery in live cells

Strains WT (pcz1), *dfi1/DFI1-GFP* (pcz12) and *dfi1/dfi1*$_{G273,\ 277L}$*-GFP* (pcz23) were grown on the surface of YPD plates at 30°C (panels 1–4) or in liquid medium (panels 5–8), washed, and visualized using a Nikon microscope at 60×. Lower panels, bright field (BF); upper panels GFP fluorescence (F). Panels 1, 2: WT (untagged); panels 3–6: *dfi1/DFI1-GFP*; panels 7, 8: *dfi1/dfi1*$_{G273,\ 277L}$*-GFP*.

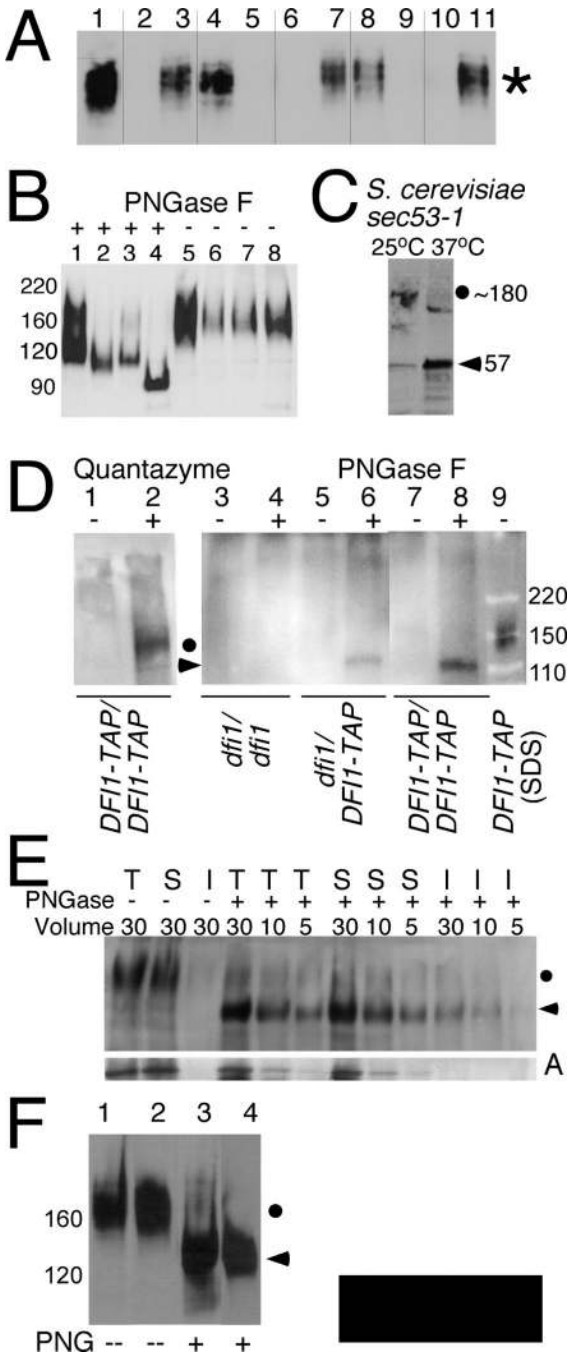

Fig. 7. Dfi1p is found in a membrane fraction and some Dfi1p molecules are covalently linked to the cell wall

A. Dfi1p-TAP fractionates to a membrane fraction. Exponentially growing cells of strain *DFI1-TAP/DFI1-TAP* (pcz11) were extracted and membranes were collected as described in Experimental Procedures. After treatment in buffers described below, membranes were pelleted as described in Experimental Procedures. The supernatants and pellets were immunoblotted with anti-HA monoclonal. Lane1: total extract; lane 2: treatment with extraction buffer (EB) only, supernatant; lane 3: treatment with extraction buffer (EB) only, pellet; lane 4: EB 1% Triton X-100, supernatant; lane 5: EB 1% Triton X-100, pellet; lane 6: EB 4M urea, supernatant; lane 7: EB 4M urea, pellet; lane 8: EB 4M urea Triton X-100,

supernatant; lane 9: EB 4M urea Triton X-100, pellet; lane 10: EB 0.1M $NaCO_3$, supernatant; lane 11: EB 0.1M $NaCO_3$, pellet. * indicates Dfi1p-HA.

B. Dfi1p-TAP is a glycosylated protein. Exponentially growing cultures of strains pcz11 (*DFI1-TAP/DFI1-TAP*), pcz19 (*mnt1/mnt1, DFI1/DFI1-TAP*), pcz20 (*mnt2/mnt2, DFI1/DFI1-TAP*), pcz21 (*mnt1/mnt1 mnt2/mnt2, DFI1/DFI1-TAP*) were grown in YPD. Equal amounts of total protein were incubated for 1 hour at 37°C in the presence (lanes 1–4) or absence (lanes 5–8) of 1500U of PNGase F (New England Biolabs). Lanes 1, and 5 correspond to *DFI1-TAP/DFI1-TAP* cell extracts; lanes 2, and 6: *mnt1/mnt1, DFI1/DFI1-TAP*; lanes 3, and 7: *mnt2/mnt2, DFI1/DFI1-TAP*; lanes 4, and 8: *mnt1/mnt1 mnt2/mnt2, DFI1/DFI1-TAP* double nulls. Dfi1p levels correlated with *DFI1-TAP* gene dosage. Immunoblot was probed with anti-HA. Numbers at left indicate mobilities of molecular weight markers.

C. Unglycosylated Dfi1p migrates aberrantly on SDS-PAGE. *S. cerevisiae sec53-1* strain bearing plasmid pYES*DFI1-HA* was grown in CM-ura at the permissive (25°C) or restrictive temperature (37°C) for 1 hour, after which protein was extracted in RIPA buffer and processed for immunoblotting. Numbers at right indicate the apparent molecular weights of Dfi1p species. The dot indicates WT Dfi1p-HA and the arrowhead indicates deglycosylated Dfi1p-HA.

D. Dfi1p-TAP is crosslinked to the cell wall. Cells from strain *DFI1-TAP/DFI1-TAP* (pcz11) were fractionated as described in (Pitarch et al., 2008) to isolate cell walls. The cell wall fraction was incubated in the presence (lane 2) or absence (lane1) of Quantazyme beta glucanase and released material was analyzed by immunoblotting with anti-HA monoclonal. For lanes 3–9, cell wall fractions from strains pcz5 (*dfi1* null, lanes 3 and 4), pcz9 (*dfi1/DFI1-TAP,* lanes 5 and 6), and pcz11 (*DFI1-TAP/DFI1-TAP*, lanes 7 and 8) were incubated in the presence (even lanes) or absence (odd lanes) of PNGase F endoglycosidase and the released material was analyzed by immunoblotting with anti-HA monoclonal. Lane 9 contains a mixture of molecular weight markers and undigested Dfi1p extracted from whole cells with SDS. Dot indicates mobility of Dfi1p-TAP; arrowhead indicates mobility of PNGase-digested Dfi1p-TAP, lacking N-linked oligosaccharides; numbers at right indicate mobility of molecular weight markers.

E. Relative amounts of detergent and PNGase extractable Dfi1p-TAP. Cells were extracted as described in Experimental Procedures. The Total, Soluble and Insoluble fractions were either digested or not digested with PNGase F. Different volumes of sample were analyzed by SDS PAGE and immunoblotting to allow comparisons of quantities of Dfi1p-HA in the various fractions. Upper panel probed with anti-HA. Lower panel probed with anti-actin. Lanes were (left to right): 1, Total, undigested, 30 µl; 2, Soluble, undigested, 30 µl; 3, Insoluble, undigested, 30 µl; 4, Total, digested, 30µl; 5, Total, digested, 10 µl; 6, Total, digested, 5 µl; 7, Soluble, digested, 30 µl; 8, Soluble, digested, 10 µl; 9, Soluble, digested, 5 µl; 10, Insoluble, digested, 30 µl; 11, Insoluble, digested, 10 µl; 12, Insoluble, digested, 5 µl. Dot indicates mobility of Dfi1p-TAP; arrowhead indicates mobility of PNGase F-digested Dfi1p-TAP; A, actin; T, total; S, soluble fraction; I, insoluble fraction.

F. Normal levels of $Dfi1p_{G273,\ 277L}$-TAP protein. Cells of strains *dfi1/DFI1-TAP* (pcz9) or *dfi1/dfi1*$_{G273,\ 277L}$*-TAP* (pcz22) were extracted in RIPA buffer and extracts were treated with or without PNGase F endoglycosidase and analyzed by SDS-PAGE and Western blotting with anti-HA. Numbers at left indicate mobility of markers. Lanes were: 1, *DFI1-TAP*, no enzyme; 2, *dfi1*$_{G273,\ 277L}$*-TAP*, no enzyme; 3, *DFI1-TAP*, with PNGase F; 4, *dfi1*$_{G273,\ 277L}$*-TAP*, with PNGase F. Dot indicates mobility of Dfi1p-TAP; arrowhead indicates mobility of PNGase F-digested Dfi1p-TAP.

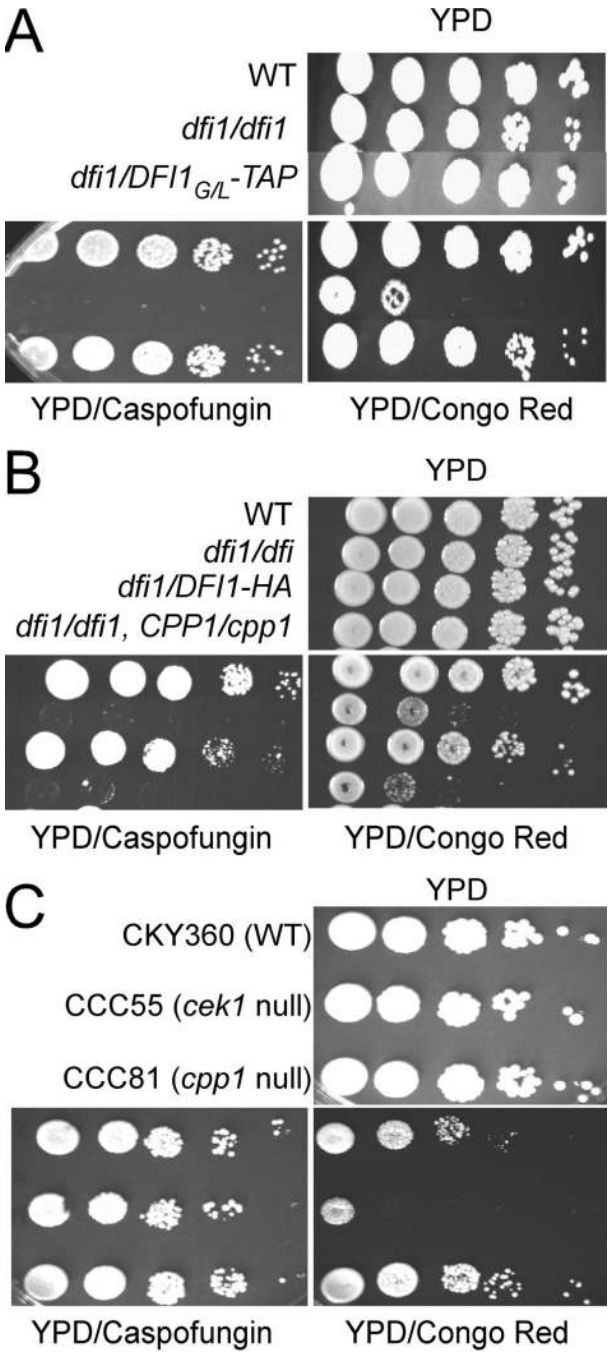

Fig. 8. Dfi1p is a functionally important component of the cell wall

A. Exponentially growing cells of strains WT (pcz1), *dfi1/dfi1* null (pcz5), and *dfi1/dfi1*$_{G273,\ 277L}$*-TAP* (pcz22) were serially diluted, plated on YPD (top panel), YPD 90 ng/ml Capsofungin (bottom panel, left), or YPD 200 μg/ml Congo Red (bottom panel, right) and incubated at 30°C for 30 hrs (YPD or YPD+Congo Red) or 48 hrs (YPD+caspofungin).

B. Exponentially growing cells of strains WT (pcz1), *dfi1/dfi1* null (pcz5), *dfi1/DFI1-HA* (pcz8), and *dfi1/dfi1, cpp1/CPP1* (pcz6) were serially diluted, plated on YPD (top panel), YPD 90 ng/ml Capsofungin (bottom panel, left), or YPD 200 μg/ml Congo Red (bottom panel, right) and incubated at 30°C for 30 hrs (YPD or YPD+Congo Red) or 48 hrs (YPD +caspofungin).

C. Exponentially growing cultures of CKY360 (CAI4, URA3), *cek1/cek1* (CCC55), and *cpp1/cpp1* (CCC81) were serially diluted, plated on YPD (top panel), YPD 150ng/ml Capsofungin (bottom panel, left), or YPD 250 μg/ml Congo Red (bottom panel, right) and incubated at 30°C for 48 hrs.

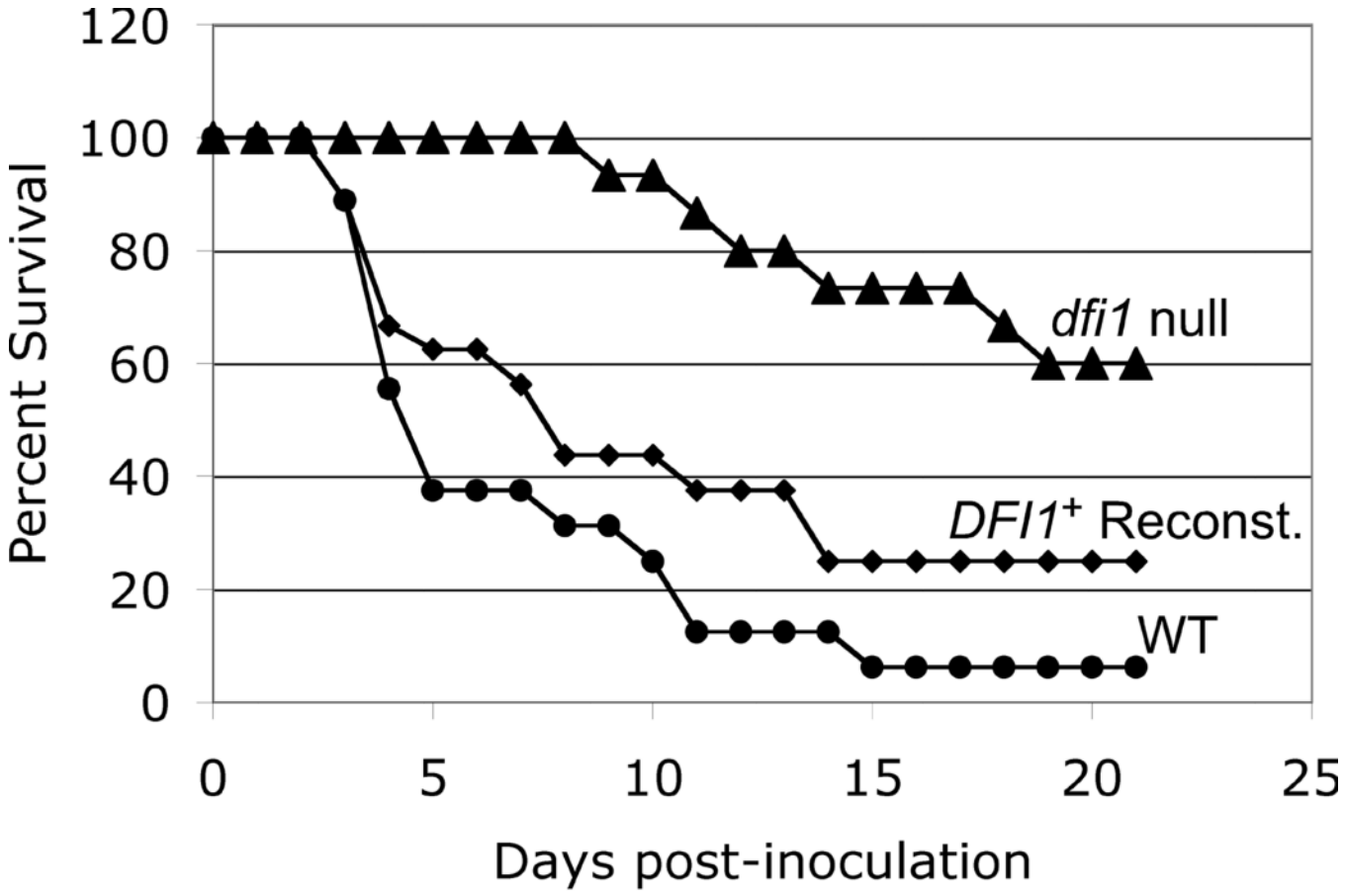

Fig. 9. Attenuated virulence of the *dfi1* null mutant

Female CF-1 mice were inoculated via the lateral tail vein with 3×10^5 cells of *C. albicans* strains. Mice were sacrificed when moribund. Two experiments with a total of 16 mice per strain were conducted; combined results are shown. Survival curves for mice inoculated with WT and *dfi1* null mutant were significantly different ($p<0.001$ log rank test with Sidak correction for multiple comparisons). Strains were: circles, WT (pcz24); triangles, *dfi1* null mutant (pcz25); diamonds, *DFI1*$^+$ reconstituted strain (pcz26).

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Table 1

Phenotypes of mutant strains lacking the following putative membrane or cell wall proteins.

Orf19.#	CGD name	Description	Invasive Filamentation[a]	Congo Red[b]	Calcofluor White[b]
NA	NA	WT parental strain	5+	4+	4+
orf19.5537		homolog of *S. cerevisiae WSC1/3*	5+	4+	3+
orf19.5867	*WSC1*	homolog of *S. cerevisiae WSC1/3*	5+	4+	4+
orf19.4772	*SHO1*	homolog of *S. cerevisiae SHO1*	4+	2+*	4+
orf19.1490	*MSB2*	homolog of *S. cerevisiae MSB2*	3+	3+*	4+
orf19.207		Glycosylated, GPI, large	5+	4+	4+
orf19.7084	*DFI1*	Defective Filamentous Invasion	1+	1+*	1+*
orf19.4906		*DFI1*-like	5+	4+	3+*
orf19.1488		Four transmembrane domains	5+	4+	4+

[a] 5+ indicates production of invasive filaments is like WT strain. 3+ or 1+ indicates fewer or no filaments when compared to WT strain.

[b] 4+ indicates normal resistance to drugs; 1+ indicates 10^3 fold more sensitivity to drugs.

* Drug sensitivity was not fully remediated by sorbitol.

Table 2

C. albicans strains used in this study

Strain	Description	Genotype	Source
BWP17		SC5314 *ura3*Δ::*imm434/ura3*Δ::*imm434 his1::hisG/his1::hisG arg4::hisG/ arg4::hisG*	(Wilson et al., 1999)
CKY360		CAI4, *ADE2/ade2::*pDBI52 (Ura$^+$)	Lab collection
CCC55	*cek1* null	CAI4, *cek1*Δ*::hisG/cek1*Δ*::(hisG-URA3-hisG)*	(Csank et al., 1997)
CCC81	*cpp1* null	CAI4, *cpp1*Δ*::hisG/cpp1*Δ*::(hisG-URA3-hisG)*	(Csank et al., 1998)
pcz1	Wild type	BWP17, *ura3*Δ::*imm434/URA3*	This work
pcz2	Heterozygote null	BWP17, *DFI1/dfi1*Δ::SAT flipper	This work
pcz3		pcz2, *dfi1*Δ::*URA3* flipper/*dfi1*Δ::SAT flipper	This work
pcz4		pcz3, *dfi1Δ::*FRT/*dfi1Δ::*FRT	This work
pcz5	*dfi1* null	pcz4, *ura3*Δ::*imm434/URA3*	This work
pcz6	*dfi1/dfi1 CPP1/cpp1*	pcz5, *CPP1/cpp1*Δ::SAT flipper	This work
pcz7	Complemented	pcz5, *dfi1*Δ*/dfi1*::*DFI1*	This work
pcz8	*DFI1-HA*	pcz5, *dfi1*Δ*/dfi1*::*DFI1-HA*-SAT placer	This work
pcz9	*DFI1-TAP*	pcz5, *dfi1*Δ*/dfi1*::*DFI1-His$_6$HA-HIS* placer	This work
pcz10		pcz9, *DFI1-TAP-HIS* placer/*DFI1-TAP*-SAT placer	This work
pcz11	*DFI1-TAP/DFI1-TAP*	pcz9, *DFI1-TAP-HIS* placer/*DFI1-TAP*	This work
pcz12	*DFI1-GFP*	pcz5, *dfi1*Δ */dfi1:: DFI1-GFP*-SAT placer	This work
pcz22	*dfi1*$_{G273,\ 277L}$*-TAP*	pcz5, *dfi1*Δ */dfi1*::*dfi1*$_{G273,\ 277L}$*-TAP*-SAT placer	This work
pcz23	*dfi1*$_{G273,\ 277L}$*-GFP*	pcz5, *dfi1*Δ */dfi1*::*dfi1*$_{G273,\ 277L}$*-GFP*-SAT placer	This work
pcz24	WT	pcz1, *his1Δ/HIS1*$^+$ *arg4Δ/ARG4*$^+$	This work
pcz25	*dfi1* null	pcz5, *his1Δ/HIS1*$^+$ *arg4Δ/ARG4*$^+$	This work
pcz26	Complemented	pcz25, *dfi1*Δ*/dfi1*:: *DFI1-HA*-SAT placer	This work