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Fuz1, a MYND domain protein, is required for cell morphogenesis in *Ustilago maydis*

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Abstract

Ustilago maydis is a Basidiomycete fungus that exhibits a yeast-like nonpathogenic form and a dikaryotic filamentous pathogenic form. Generation of these two forms is controlled by two mating type loci, *a* and *b*. The fungus undergoes additional morphological transitions in the plant that result in formation of a third cell type, the teliospore. The *fuz1* gene is necessary for this developmental program. Here we report cloning and sequencing of *fuz1* and show that it contains an open reading frame with coding capacity for a protein of 1421 amino acids. The Fuz1 protein belongs to the family of MYND Zn finger domain proteins. We generate a null mutation in strains of opposite mating type and show that *fuz1* is necessary for conjugation tube formation, a morphological transition that occurs in response to pheromones. We generate *fuz1*[−] diploid strains heterozygous at *a* and *b* and show that *fuz1* is also necessary for postfusion events (maintenance of filamentous growth). We also demonstrate that *fuz1* is necessary for cell morphogenesis of the yeast-like cell: normal cell length, location and number of septa, cell separation and constriction of the neck region. Fuz1 is also required for cell wall integrity and to prevent secretion of a dark pigment. We propose that the MYND domain may interact with different proteins to regulate cell morphogenesis.

Keywords

cell separation; conjugation tube; filamentous growth; pigment production; septum location

INTRODUCTION

Ustilago maydis is a Basidiomycete fungus that exhibits two basic morphologies: a yeast-like and a filamentous dikaryotic form and is capable of switching from one to the other (the dimorphic switch) (reviewed in Banuett 1995, 2002; Feldbrügge et al 2004, Klosterman et al 2007). The dimorphic switch is crucial to pathogenicity; the yeast-like form is nonpathogenic whereas the filamentous dikaryon is pathogenic. Growth of the filamentous pathogenic dikaryon leads to formation of tumors in its hosts, maize (*Zea mays* L) and teosinte (*Zea mays* ssp. *parviglumis* and spp. *mexicana*) (reviewed in Banuett 2002, Christensen 1963, Klosterman et al 2007). In addition to the dimorphic switch, the fungus undergoes extensive morphological changes within the tumors that result in formation of a distinct cell type, the teliospore (Banuett and Herskowitz 1996, Snetselaar and Mims 1994). Teliospores undergo meiosis to produce the haploid phase (reviewed in Christensen 1963, Banuett 2002, Klosterman

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et al 2007). Because the developmental program leading to teliospore formation does not occur outside the plant it has been hypothesized that plant signals trigger this pathway (Banuett and Herskowitz 1996, Regenfelder et al 1997, Ruiz-Herrera et al 1999).

The dimorphic switch is genetically programmed by two mating type loci (*a* and *b*) and by environmental conditions (reviewed in Banuett 1995, 2002; Feldbrügge et al 2004, Kahmann and Schirawski 2007, Klosterman et al 2007). The *a* locus has two alleles, *a1* and *a2* (Rowell and DeVay 1954); each contains a pheromone precursor and pheromone receptor gene (Bölker et al 1992) that regulate conjugation tube formation and cell fusion of haploid strains (Banuett and Herskowitz 1994a, b; Snetselaar et al 1996, Spellig et al 1994) and filamentous growth on charcoal agar (Banuett and Herskowitz 1989, Bölker et al 1992, Spellig et al 1994) but not in planta (Banuett and Herskowitz 1996, Regenfelder et al 1997). The *b* locus is multiallelic with at least 25 naturally occurring alleles (Puhalla 1970, Rowell and DeVay 1954; reviewed in Banuett 2007, Holliday 1974, Klosterman et al 2007). Each allele contains two genes, *bW* and *bE*, that code for homeodomain proteins (Gillissen et al 1992, Kronstad and Leong 1990, Schulz et al 1990). The *bW* and *bE* polypeptides coded by different *b* alleles interact to form a combinatorial activity (*bW_x-bE_y*) that regulates gene expression in the filamentous dikaryon (Babu et al 2005, Brachmann et al 2001, Kämper et al 1995, Nugent et al 2004, Yee and Kronstad 1998; reviewed in Banuett 2007, Kahmann and Schirawski 2007). The *b* locus is the major determinant of pathogenicity and filamentous growth and also is implicated in control of meiosis (Day et al 1971, Puhalla 1970, Rowell and DeVay 1954; reviewed in Banuett 2007, Kahmann and Schirawski 2007).

The yeast-like unicellular haploid form is elongated, approximately 15–18 µm long and 5 µm wide and forms a bud once per cell cycle at one of the cell poles, slightly off center (Banuett and Herskowitz 2002, Holliday 1974, Jacobs et al 1994). *U. maydis* yeast-like cells can use the same bud site repeatedly as in apiculate yeasts or they can choose a new bud site at the same or opposite pole (Jacobs et al 1994), but the mechanisms by which one pole is chosen versus the other remain unknown. The bud grows by incorporation of new cell wall material at the tip with no phase of isotropic growth (Banuett and Herskowitz 2002). The final shape of the bud results from a progression of morphological changes, from an initial cylindrical or conical structure to a bulbous intermediate and finally to the elongated cell with tapered ends (Banuett and Herskowitz 2002). In large-budded cells before cell separation, two septa are present, one on the mother side of the neck region and the other on the bud side of the neck region. The septum on the mother side forms before that in the daughter cell (Banuett and Herskowitz 2002, O'Donnell and McLaughlin 1984, Weinzierl et al 2002). These two septa are separated by a zone of less densely staining cell wall material, the fragmentation zone, where cell separation occurs (Banuett and Herskowitz 2002, O'Donnell and McLaughlin 1984, Weinzierl et al 2002). The actin and microtubule cytoskeleton have been implicated in bud morphogenesis (Banuett and Herskowitz 2002, Steinberg et al 2001).

The *fuz1* gene was isolated in a screen for mutants unable to form filaments when mated with a wild type strain of opposite mating type (Banuett, 1991). The *fuz1* gene is unlinked to the mating type loci (Banuett 1991). In plant inoculations with wild type and *fuz1* mutant strains of opposite mating type (for example, *a1 b1 fuz1*⁺ + *a2 b2 fuz1*[−]) tumor response and teliospore production are no different than those observed in inoculations with wild type strains. In contrast, in inoculations with *fuz1* mutants of opposite mating type (for example, *a1 b1 fuz1*[−] + *a2 b2 fuz1*[−]), the tumors are small, white, restricted in location and do not produce teliospores; thus the sexual cycle cannot be completed (Banuett and Herskowitz 1996). A time course comparison of the infectious process between wild type strains (for example, *a1 b1* + *a2 b2*) with those between *fuz1* mutant strains of opposite mating type indicates that the *fuz1* mutant strains can form filaments in the plant and these filaments do not differ in appearance from those observed in wild type inoculations (Banuett and Herskowitz 1996).

Teliospore formation involves a discrete developmental pathway in which hyphae become embedded in a mucilaginous matrix and then fragment and release cylindrical cells that undergo cell rounding to become the teliospores (Banuett and Herskowitz 1996; reviewed in Banuett 2002, Klosterman et al 2007). Interestingly, infections with *fuz1* mutant strains of opposite mating type cause uniform arrest of the teliospore developmental program at the hyphal fragmentation stage (Banuett and Herskowitz 1996). The *fuz1* mutant hyphae are unable to fragment, become aberrantly shaped and exhibit abnormal cytoplasmic protrusions not observed in wild type hyphae (Banuett and Herskowitz 1996).

These observations prompted us to examine the role of *fuz1* in cell morphogenesis of the yeast-like cell and in the dimorphic switch. Here we report the cloning of the *fuz1* gene and show that *fuz1* codes for a MYND Zn finger domain protein that is required for the morphological differentiation in response to pheromones, for events at the junction of cells, for cell wall integrity and to control release of a dark pigment.

MATERIALS AND METHODS

Strains, media, and growth conditions

U. maydis strains used in this study are listed (Table I). YEPS (Tsukuda et al 1988) was used for routine growth of *U. maydis* strains. UMC broth (*U. maydis* complete, Holliday 1974) was used for growth of strains to be tested on charcoal agar (the fuzz reaction, Banuett and Herskowitz 1989). Charcoal agar is as described (Holliday 1974). Strains to be tested on charcoal agar were cross-streaked against standard tester strains (*a1 b1* [FB1], *a2 b2* [FB2], *a2 b1* [FB6a], and *a1 b2* [FB6b]) or spotted and compared to diploids (*a1/a2 b1/b2* [FBD-12] and *a1/a2 b1/b1* [FBD12-3]) (Banuett and Herskowitz 1989). Plates were sealed with Parafilm and incubated at room temperature. For replica mating 0.3 mL of an overnight culture of *a1 b1* (FB1) were spread on charcoal agar medium (Banuett and Herskowitz 1989, Banuett 1991) onto which 2 to 3 d old colonies of *a2 b2 fuz1⁻* (FBf10-1i) transformants, grown on YEPS agar at 28 C, were replica mated. YEPS 1 M sorbitol supplemented with hygromycin B (200 µg/mL) or carboxin (2 µg/mL) or with both drugs was used respectively for selection of transformants or *fuz1⁻* diploids. Transformants and diploids were purified on the same medium without sorbitol.

Bacterial strains were grown on Luria-Bertani medium supplemented with ampicillin (50 µg/mL) and X-gal (20 µg/ml) for selection and screening of transformants, respectively.

Enzymes and reagents

Restriction endonucleases were from Roche or New England BioLabs; Expand Polymerase, DIG-labeling kit, RNase A and Southern analysis buffers from Roche; AmpliTaq and dNTPs from ABI; chemicals for the preparation of buffers and media from Sigma and Fisher Scientific Co.; DNeasy Plant DNA kit for isolation of genomic DNA and QIAquick Gel extraction Kit from QIAGEN; Hygromycin B from Calbiochem; lysing enzymes, Congo Red, thiabendazole, Calcofluor 28, and DAPI (4',6'-diamidino-2-phenylindole-dihydrochloride) from Sigma; Ampicillin from US Biological; and X-Gal from Gold Biotechnology. Oligonucleotides were synthesized by InVitrogen.

Light microscopy

Cells were observed with Nomarski interference and epifluorescence optics with a BX61 Olympus microscope equipped with an ORCA CCD camera (Hamamatsu) and images captured with Simple PCI imaging software (Scientific Instruments, San Francisco, California), and processed with Adobe Photoshop 7.0.

Cell wall, septum and nuclear staining

Procedures used were described in Banuett and Herskowitz 2002.

Growth of *fuz1* mutant and wild type strains on different media

Fuz1 mutant (FBf10-1i, FBf10-3c, FB18, FB19, FB20, FB21) and wild type strains (FB1, FB2) (Table I) were grown in YEPS broth until saturation. Tenfold serial dilutions of each strain were prepared in a 96-well microtiter plate and an aliquot of each dilution for each strain spotted onto YEPS agar and YEPS agar containing 1M sorbitol, 1M KCl, 1M NaCl, 1.2M NaCl, 10 µg/mL or 20 µg/mL thiabendazole, Calcofluor white (40 µg/mL) or Congo red (50 µg/mL). Strains also were streaked on the same media. The plates were incubated at 28 C and colony growth and morphology were recorded over several days and photographed with a Nikon digital camera. At least three independent tests were carried out on a given day on three different days.

DNA-mediated transformation of *U. maydis*

Strains grown in YEPS broth were transformed according to Fotheringham and Holloman (1989) or Wang et al (1988).

Cloning *fuz1* by functional complementation

Strain FBf101i (*a2 b2 fuzI*⁻) (Banuett 1991; Table I) was transformed with a cosmid library and approximately 3000 hygromycin resistant transformants were replica mated onto a lawn of *al b1* (FB1) cells on charcoal agar medium, as described above, and screened for colonies that form filaments with the wild type strain (*Fuz*⁺ phenotype in a *Fuz*⁻ background, Fig. 1A). *Fuz*⁺ colonies were purified, grown in UMC broth overnight and cross-streaked on charcoal agar versus standard tester strains FB1 (*a1 b1*) and FB2 (*a2 b2*) (TABLE I). As controls FB1 was streaked versus FB2 and FB1 and FBf10-1i versus FB1 and FB2. Genomic DNA from two *Fuz*⁺ candidate strains (37a-3 and 38a-11) was obtained as described in Wang et al (1988) and Banuett and Herskowitz (1994) to recover the integrated cosmid and flanking DNA and treated with restriction endonucleases BglII, BamHI or StuI, which do not cut within the vector (pBR322), self-ligated, and an aliquot was used to transform *E. coli* DH5α and DH5α mcr strains (Invitrogen).

Cosmid DNA was isolated from the *E. coli* transformants according to Sambrook and Russell (2001) and treated with restriction endonuclease BamHI. Seven plasmids, derived from treatment with the above restriction enzymes, were analyzed; three contain identical inserts, whereas the rest have different inserts. Nucleotide sequence of the vector-junction was generated from three different plasmids with a vector-derived primer. This sequence information was used to generate a 43-mer insert-specific oligonucleotide (FB75), which was labeled with T4 polynucleotide kinase as described in Sambrook and Russell (2001) and used for colony hybridization of *E. coli* DH5α cells transformed with the cosmid subpool from which the original *fuzI*⁺ transformants had been obtained. Cosmid DNA was isolated from 10 *E. coli* colonies that produced a positive signal, and one of the cosmids was shown to complement the *fuzI*⁻ mutation after introduction into strains FBf10-1i (*a2 b2 fuzI*⁻) and FBf10-3c (*a1 b1 fuzI*⁻). The *fuz1* cosmid was designated p12.

Subcloning *fuz1* from cosmid p12

Treatment of cosmid p12 with restriction enzyme BamHI generated 4 fragments of approximately 20, 12, 9 and 5 kb. The 12, 9 and 5 kb fragments were isolated and ligated into BamHI-treated plasmid pHL1 (Wang et al 1988) to generate plasmids p12-F12, p12-F36 and p12-F1 respectively. These plasmids were transformed into *U. maydis* strain FBf10-1i (*a2 b2 fuzI*⁻) and hygromycin resistant transformants were cross-streaked on charcoal agar versus

tester strains as described above. Transformants that contained p12-F1 produced a positive fuzz reaction with tester strain *a1 b1* (FB1) but not with *a2 b2* (FB2), whereas transformants with p12-F12 and p12-F36 did not. The 5 kb BamHI fragment also was ligated into pUC19 (BRL/InVitrogen) to generate p19F1, which was used to generate an internal deletion of the ORF (see below).

DNA sequencing and sequence analysis

Double-strand sequencing was performed with the Sequenase 2.0 kit with universal and custom-synthesized primers. The templates (pUC derived plasmids or a pBR322-derived cosmid) were denatured by standard procedures. The sequence of the 5 kb BamHI fragment in p19F1, described above, contains an open reading frame of approximately 1800 nucleotides that is truncated at its 3' end (Fig. 2A), which might explain why plasmid p12-F1 results in ~45% Fuz⁺ colonies upon transformation of the *a2 b2 fuz1⁻* strain. The remaining sequence was generated with cosmid p12 as template. The derived amino acid sequence of the putative Fuz1 protein was compared with proteins in the NCBI database with BLAST analysis (<http://www.ncbi.nlm.nih.gov/blast>) (Altschul et al 1997). Sequence alignment was carried out with MultAlin (Corpet 1988) and Clustal W (Thompson et al 1994).

Deletion of the ORF and of an internal ORF fragment

The entire ORF was deleted according to the procedure of Brachmann et al (2004) and also using a PCR-ligation free method (J. Shorts, Y. Aweiss and F. Banuett pers comm). A 1.5 kb fragment from the 5' UTR region immediately upstream of the ORF was amplified from genomic DNA by PCR with primers FBU130 (5'GTTAATTAACGCGCGCTGCGAAGCTCAAGCC) and FBU131 (5'GAATGGCCATCTAGGCCTACCACAGGAGGGAGAATGTC) (Fig. 2B). Similarly a 1.5 kb fragment from the 3' UTR region immediately downstream of the ORF was amplified with primers FBU132 (5'GAATGGCCTGAGTGGCCTACCGCGATTCCATTAGCATG) and FBU133 (5'GGTTAATTAAGGTGCTGCAGCAAAGGCGGCGG) (Fig. 2B). Primers FBU130 and FBU 133 introduce a PacI restriction enzyme site (underlined) and primers FBU131 and FBU132 a modified SfiI restriction site (underlined) (see Brachmann et al 2004). The amplified fragments were cloned into pCR2.1 TOPO (InVitrogen) to generate respectively plasmids pCR5F1 and pCR3F1. Orientation of the 5' and 3' UTR fragments in these plasmids was confirmed by restriction enzyme analysis with BsaI and XcmI, respectively. Plasmids pCR5F1 and pCR3F1 were treated with SfiI and XbaI, fractionated on a 0.7% agarose gel, and the 1.75 kb fragment from pCR5F1 and the 5.5 kb fragment from pCR3F1 were gel purified and ligated with the SfiI hygromycin cassette from plasmid pMF-1h (Brachmann et al 2004) to generate pCR2.1 TOPO containing the 5'UTR-hygB-3'UTR targeting fragment that lacks the entire ORF (pCR-Δ*fuz1*). The targeting fragment was released by treatment with restriction endonuclease PacI, gel purified after fractionation on a 1% agarose gel, introduced into haploid strains *a1 b1* (FB1) and *a2 b2* (FB2) and transformants selected as described above.

Deletion of the ORF was ascertained by PCR with primers FBU108 (5' AGCGTGTCTGTCATGTGC) and FBU114 (5' CTCGCTGGTAGTTACCAC) or FBU107 (5' AGACCGAATGGAGGATCG) and FBU115 (5' GGAGGAGATGACTACCTCG) (Fig. 2B). Primer FBU108 is upstream of FBU130; FBU107 is downstream of FBU133; and FBU114 and FBU115 are within the *hygB* gene (Fig. 2B). PCR amplification with the above sets of primers generates a 2.3 kb fragment in the null mutant strains and no fragment in wild type strains (data not shown). The presence of the ORF was detected with ORF-specific primers FBU011 (5' TGCGCCTTGACGTCGATGCCG) and FBU180 (5' GGCGCGCTTAATTAAGAGCTCCATGCTAATGGAATCGCG) (Fig. 2B), which amplify a 1.5 kb fragment in wild type strains and no fragment in the null strains (data not shown). This analysis was followed by Southern hybridization as described below.

To generate a 459 bp internal deletion of the *fuz1* ORF, plasmid p19F1 (pUC19 containing a 5 kb BamHI *fuz1* complementing fragment) was treated with XhoI, which cuts twice within the *fuz1* ORF, to generate fragments of approximately 6.5 kb and 459 bp. The 6.5 kb fragment was gel purified, and ligated with a 2.0 kb Xho-SalI fragment containing the hygromycin cassette from plasmid pHL1 (Wang et al 1988) to give rise to plasmid p19F1 Δ XhoI:: *hygB*. This internal deletion leaves 504 nucleotides at the 5' end of the ORF and 3300 nucleotides for the remainder of the ORF and replaces the deleted fragment with the hygromycin cassette. A BamHI fragment containing the construct was introduced into strain *a1 b1* (FB1). Thirty-two hygromycin resistant transformants were tested for the Fuz phenotype on charcoal agar (see MATERIALS AND METHODS) and for other phenotypes (see RESULTS). Twenty-three transformants exhibited a Fuz⁻ phenotype and the remaining nine were Fuz⁺ (data not shown).

Isolation of genomic DNA and Southern hybridization analysis

Protoplasts for isolation of genomic DNA were prepared from 10–50 mL cultures as described (Wang et al 1988, see also Banuett and Herskowitz 1994a) and subjected to the procedure described in the DNeasy Plant DNA kit (QIAGEN). For Southern hybridization analysis, genomic DNA from Fuz⁻ and Fuz⁺ transformants, and from the parental wild type strains (FB2 and FB1) (TABLE I) was digested with restriction endonuclease MscI, fractionated on a 0.7% agarose gel and transferred to an Immobilon membrane (Millipore). Conditions for transfer were as described in Sambrook and Russell (2001) and the manufacturers of the membrane (Millipore) and the DIG-labeling kit (Roche). The membrane was probed with a 520 bp DIG-labeled PCR fragment from the *fuz1* 5' UTR region (see below). The membrane was reprobed with a 872 bp DIG-labeled PCR fragment from the *HygB* gene in pHL1 (Wang et al 1988) (see below). In some cases the membrane also was reprobed with a 1.5 kb DIG-labeled PCR fragment from the ORF region. For Southern hybridization analysis of the internal deletion mutant, DNA was digested with BamHI, fractionated, and transferred as above and probed with a 1.8 kb DIG-labeled BstEII probe (see below). In control strains (FB1 and FB2) a 4.5 kb band is detected, whereas in the Fuz⁻ transformants a 6.0 kb band (data not shown). Fuz⁺ transformants contain the wild type band and an additional band indicative of a nonhomologous integration event (data not shown).

Labeling of DNA probes for Southern hybridization

The following probes were labeled with the PCR DIG-labeling kit (Roche) (see Fig. 2B): a 520 bp fragment from the *fuz1* 5'UTR region with primers FBU145 (5' CTCACGACTTGACTCACACG) and FBU146 (5'GCCGAGGATGATGCTGAATG); a 872 bp fragment from the *hygB* gene with primers FBU79 (5' GCGAGTACTTCTACACAG) and FBU80 (5' GCTTTCAGCTTCGATGTAGG); and a 1.5 kb fragment from the ORF region with primers FBU011 and FBU180, described above. A BstEII fragment that encompasses part of the ORF and the 5'UTR region was labeled randomly and used in the analysis of the internal deletion mutant. PCR fragment probes were gel purified with the QIAquick Gel extraction kit (QIAGEN) before use.

Generation of a full length *fuz1*-complementing plasmid

A 9.2 kb fragment containing the entire *fuz1* ORF (~4.3 kb) and flanking regions (~3 kb of 5' and ~2 kb of 3') was amplified from genomic DNA with primers FBU302 (5' GAGTCTAGAGCTGCGGCTTTGTAGTTG) and FBU303 (5' GAGAAGCTTCCAGCCAAGATCATCCAG), which respectively introduce XbaI and HindIII sites (underlined). The amplified fragment was gel purified, cloned into pCM619 (Kojic and Holloman 2000) to generate pCM-*fuz1* and introduced into strains *a2 b2 fuz1*⁻ (FBf10-1i), *a2 b2 Δfuz1* (FB18), and *a1 b1 Δfuz1* (FB21) with selection for carboxin-resistant transformants. Purified transformants were tested for complementation of the different

phenotypes conferred by the *fuz1*[−] mutation. All phenotypes were rescued by this autonomously replicating plasmid (data not shown).

PCR amplifications

PCR amplifications were carried out in a MJR (MJ Research Inc., USA) or Biometra (Biometra biomedizinische Analytik GmbH, Göttingen, Germany) thermal cycler. For amplification of the *fuz1* 5' and 3'UTR regions, 25 cycles (15 s denaturation at 94 C, 30 s annealing at 58–62 C, and 2 min extension at 68 C) were carried out with Expand Polymerase (Roche) and 50–100 ng of genomic DNA. For amplification of the entire ORF and flanking regions (~9.2 kb in total), 50–100 ng of genomic DNA were subjected to 25 cycles (15 s at 94 C, 30 s annealing at 61 C and 8 min extension at 68 C with Expand Polymerase [Roche]). For amplification of genomic DNA from hygromycin resistant transformants, 100 ng of genomic DNA were subjected to 30 cycles (30 s denaturation at 94 C, 30 s annealing at 55–62 C, and 45–120 s extension at 72 C) with AmpliTaq (ABI). For amplification of DNA to generate DIG-labeled probes, conditions recommended by the manufacturer (Roche) were followed.

Conjugation tube assay

Conjugation tube formation was assayed as described by Banuett and Herskowitz (1994a, b). Strains were grown in YEPS until saturation. Aliquots were used to inoculate minimum medium with reduced nitrogen and 0.1% charcoal, grown till saturation, and concentrated fivefold (see Banuett and Herskowitz 1994b). An equal volume of cells (0.5 mL) from two different strains was mixed in 5 mL of fresh medium, incubated at 28 C with gentle shaking (60–80 rpm), aliquots taken at 1 h intervals beginning 2 h after mixing and continued 16–24 h. Mixtures were: i. FB1 (*a1 b1*) and FB2 (*a2 b2*); ii. FB1 and FBf10-1i (*a2 b2 fuz1*[−]); iii. FB1 and FB18 (*a2 b2 Δfuz1*); iv. FB2 and FBf10-3c (*a1 b1 fuz1*[−]); v. FB2 and FB21 (*a1 b1 Δfuz1*); (f) FBf10-1i and FBf10-3c; vi. FB18 and FB21. Formation of conjugation tubes was examined microscopically with Nomarski optics. Some samples also were stained with DAPI and examined with epifluorescence optics to ascertain nuclear position and migration. Pure cultures of each strain were grown under the same conditions.

Generation of *fuz1*[−] diploids

Strain *a1 b1 fuz1*[−] (FBf10-3c) was transformed with plasmid pCBX122 (Keon et al 1991), with selection for carboxin resistant transformants, to generate strain FB3c-cbx; strain FB2 was transformed with pCM54 (Tsukuda et al 1988), with selection for hygromycin resistant transformants, to generate strain FB2-H. Strains FB18 (*a2 b2 Δfuz1::hygB*), FB3c-cbx (*a1 b1 fuz1*[−] [pCBX122]), and FB2-H (*a2 b2* [pCM54]) were grown overnight to OD₆₀₀ 0.9–1.0 in YEPS broth supplemented with hygromycin or carboxin, protoplasts prepared as described above, and 0.2 mL protoplasts (~1 × 10⁸/mL) of FB2-H and FB3c-cbx or FB3c-cbx and FB18 were mixed and treated as for transformation. Diploids were selected on hygromycin carboxin agar (see above), colonies purified on the same medium without sorbitol, grown in UMC broth and tested for filamentous growth by spotting on charcoal agar. Their sensitivity to Congo red was determined as for *fuz1*[−] haploid strains, as described above. Heterozygosity at the *b* locus was determined by Southern hybridization analysis as described in Banuett and Herskowitz (1994a).

RESULTS

fuz1 codes for a MYND domain protein

The *fuz1* open reading frame (ORF) spans 4263 nucleotides and has a coding capacity for a protein of 1421 amino acids (Fig. 2A) with a predicted MW 153.84 KDa and a pI of 6 (GenBank accession No. EU035495; MUMDB um02587 (<http://mips.gsf.de/genre/proj/ustilago/>). The

putative Fuz1 protein contains two regions with significant similarity to other proteins in the databases (Figs. 2A, 3). A domain of 100 amino acid residues (692–791; Figs. 2A, 3) located near the middle of the protein contains a MYND-type Zn finger domain, which is conserved in diverse eukaryotic proteins (CDD, Marchler-Bauer et al 2007). The MYND domain extends from amino acid 745 to 791 (Figs. 2A,3). The residues immediately upstream of this domain are highly conserved among the fungal proteins but not among nonfungal MYND domain-containing proteins. An amino terminal domain of approximately 140 amino acid residues (Figs. 2,3) shared with other fungal proteins, some of which (*Aspergillus nidulans* SamB and *Saccharomyces cerevisiae* Mub1) are involved in fungal morphogenesis (Krüger and Fischer 1998). The function of the other fungal proteins is not known. An additional block of 26 amino acid residues is found near the middle of the protein (amino acid residues 628–653, Fig. 2A) and is shared with the same fungal proteins. The putative Fuz1 protein also contains two histidine-rich regions (residues 531–542) and (residues 568–576), and a glutamine rich region (499–577), which are not present in the other proteins (Fig. 2A). Several potential PKC and PKA phosphorylation sites and myristoylation sites are present (Prosite, Falquet et al 2002).

Generation of a *fuz1* null mutation and role in filamentous growth

To determine the *fuz1* null phenotype in several cell processes, we generated a *fuz1* null mutation by a one-step gene replacement procedure as described in MATERIALS AND METHODS. This mutation removes the entire ORF and replaces it with a 1.0 kb hygromycin cassette from plasmid pMF-1h (Fig. 2B) (see MATERIALS AND METHODS). Introduction of the targeting fragment (see MATERIALS AND METHODS) into haploid strains *a2 b2* (FB2) and *a1 b1* (FB1) and cross-streaking the transformants on charcoal agar against tester strains (see MATERIALS AND METHODS) revealed that 40% and 60% of FB2 and FB1 transformants, respectively, exhibited a Fuz[−] phenotype (no filaments or reduced filament formation) and the remaining a Fuz⁺ phenotype. The Fuz[−] transformants could be due to gene replacement by homologous recombination or de novo inactivation of another gene by nonhomologous recombination. Gene replacement was confirmed by PCR and Southern hybridization analysis (see MATERIALS AND METHODS). The latter analysis showed that a 5.5 kb band present in wild type control strains was shifted to a band of faster mobility of 2.5 kb in all Fuz[−] strains (Fig. 4A), when probed with a 5' UTR fragment, as expected for a gene replacement event, whereas the Fuz⁺ transformants contained two bands, one corresponding to the band observed in control strains (5.5 kb) and another band of different mobility than that observed in Fuz[−] strains (data not shown). These observations indicate that the wild type *fuz1* gene had been replaced by the null allele ($\Delta fuz1::hygB$) in the Fuz[−] strains, whereas the targeting fragment had integrated at nonhomologous loci in the Fuz⁺ strains (data not shown). The result was confirmed further by probing the same membrane with a *hygB*-specific fragment (Fig. 4B) or with an ORF-specific probe (data not shown) (see MATERIALS AND METHODS).

The Fuz phenotype of the *fuz1* null mutant and the mutant with an internal deletion was tested on charcoal agar (MATERIALS AND METHODS). Cross-streaking of two wild type strains (*a1 b1 fuz1*⁺ + *a2 b2 fuz1*⁺) yields a white fuzziness due to the formation of dikaryotic filaments 24 h after incubation (Fig. 1B), whereas cross-streaking of *a1 b1 fuz1*⁺ + *a2 b2 $\Delta fuz1$* yields reduced filament formation (Fig. 1B) and cross-streaking of *a1 b1 $\Delta fuz1$* + *a2 b2 $\Delta fuz1$* results in absence or further reduction of filament formation after 48–96 h incubation (Fig. 1B). Thus the *fuz1* null mutant behaves similarly to the original UV-induced mutant and to the *fuz1* mutant with a small internal deletion of the ORF (data not shown). These results are consistent with a role of *fuz1* in filament formation on charcoal agar (see Banuett 1991).

Role of *fuz1* in conjugation tube formation

Because formation of dikaryotic filaments involves cell fusion and filamentous growth of the fusion product (the dikaryon), reduced filament formation on charcoal agar (Fig. 1B) could result from a requirement of *fuz1* for either or both of these processes. Cell fusion and

filamentous growth can be assayed independently with the appropriate strains (see for example Banuett and Herskowitz 1989,1994a;Dürrenberger and Kronstad 1999,Gold et al 1994,Regenfelder et al 1997,Spellig et al 1994). Before cell fusion cells form conjugation tubes, a morphological differentiation that occurs in response to pheromones produced by cells of opposite mating type (Banuett and Herskowitz 1994a,b;Snetselaar et al 1996,Spellig et al 1994). To determine if *fuz1* is required for conjugation tube formation, we assayed this process in coculture of different strains in low nitrogen medium containing charcoal (see MATERIALS AND METHODS;Banuett and Herskowitz 1994a,b). Pure cultures of the wild type and *fuz1* mutant strains arrest as unbudded cells when grown in low nitrogen medium (Fig. 5A, L). Coculture of wild type haploid strains (*a1 b1* + *a2 b2*) resulted in emergence of conjugation tubes approximately 3.5 h after the cells were mixed (Fig. 5B, C). Greater than 90% of the cells exhibited a response (1000 cells examined) consistent with previous observations that the response is rapid—begins approximately 3 h after mixing—and is synchronous—90% of cells respond (Banuett and Herskowitz 1994a,b). The conjugation tubes exhibited the characteristic sinuous appearance (Fig. 5B, C), which is distinct from that of the dikaryotic filaments (Fig. 5D). The conjugation tubes continued to elongate and at 8 h, long, straight-growing filaments (Fig. 5D) were present and contained two nuclei in the tip cell (Fig. 5F), indicative of cell fusion and formation of the dikaryon. These filaments became more numerous with prolonged incubation time (Fig. 5E); after overnight incubation (16–24 h) a mat of white filaments was visible on the surface of the liquid (data not shown). In coculture of wild type and *fuz1*[−] or wild type and Δ *fuz1* strains carrying different *a* and *b* alleles conjugation tubes first appear at 6.5–7.5 h or later after mixing of the different strains (Fig. 5G–I), a delay of 3 or more h compared to the wild type strains. The number of cells with conjugation tubes is reduced at least 100-fold compared to that observed in coculture of wild type strains (≥ 1000 cells examined for each combination of strains). After overnight incubation reduced numbers of dikaryotic filaments are observed compared to coculture of wild type haploid strains (Fig. 5H, I). In coculture of strains that carry different *a* and *b* alleles and the same *fuz1* mutation, conjugation tube formation is reduced at least 10-fold compared to the mixture of wild type and *fuz1* mutant strains (Fig. 5J, K), that is at least 1000-fold with respect to the wild type strains. Even after overnight incubation conjugation tubes were observed rarely and few or no dikaryotic filaments were present (Fig. 5K). These observations indicate that *fuz1* is required for conjugation tube formation and for timing of this morphological response.

Role of *fuz1* in maintenance of filamentous growth

In addition to its role in conjugation tube formation *fuz1* also might be required for filamentous growth of the dikaryon. To test this possibility independently of its role in conjugation tube formation and cell fusion, we generated *fuz1*[−] diploids (*a1/a2 b1/b2 Δ fuz1/*fuz1*[−]*) and diploids heterozygous for Δ *fuz1* (*a1/a2 b1/b2 Δ fuz1/*fuz1*⁺*) (see MATERIALS AND METHODS) and spotted them on charcoal agar. The heterozygous Δ *fuz1* (*a1/a2 b1/b2 Δ fuz1/*fuz1*⁺*) strain formed mycelial colonies (Fuz⁺ phenotype) (data not shown) indistinguishable from those of diploids homozygous for *fuz1*⁺ (*a1/a2 b1/b2 *fuz1*⁺/*fuz1*⁺*) (Fig. 1Ca), whereas the *a1/a2 b1/b2 Δ fuz1/*fuz1*[−]* diploid did not form filaments (Fuz[−] phenotype) (Fig. 1Cc, d, e), similar to *a1/a2 b1/b1* diploids (FBD12-3) (Fig. 1Cb). These results indicate that *fuz1* is required for maintenance of filamentous growth, a postfusion event.

***fuz1* mutants exhibit altered colony and cell morphology**

To determine the role of *fuz1* in cell morphogenesis, we undertook a detailed analysis of colony and cell morphology of *fuz1* mutants and wild type strains. *fuz1* mutants exhibit altered colony morphology characterized by ridges (undulations) that contrast with the smoother surface of the wild type parental strain (c.f. Fig. 6C, D and 6A, B). Because altered colony morphology might be due to altered cell morphology, we determined cell morphology in asynchronous exponentially growing cultures of *fuz1* null mutant strains (*a2 b2 Δ fuz1::hygB* [FB18]; *a1*

b1 Δfuz1::hygB [FB21]) and *fuz1⁻* mutant haploid strains of different mating types containing the original UV-induced mutation (*a2 b2 fuz1⁻* [FBf10-1i], *a1 b1 fuz1⁻* [FBf10-3c], *a2 b1 fuz1⁻* [FBf10-5j], *a1 b2 fuz1⁻* [FBf10-1c]), wild type haploid strains (*a1 b1 fuz1⁺* [FB1], *a2 b2 fuz1⁺* [FB2]) and a wild type diploid strain (*a1/a2 b1/b2 fuz1⁺/fuz1⁺* [FBD-12]) (Table I) with Nomarski differential optics (see MATERIALS AND METHODS). Different fields were examined for cell length measurements. A total of 350 *fuz1⁻* cells of different mating types, 100 wild type haploid cells and 100 wild type diploid cells were analyzed during early log phase of growth. *fuz1⁻* cells, regardless of mating type, are at least 1.6× longer than the parental wild type cells (26 ± 0.7 [*fuz1⁻*] vs. 15.5 ± 0.5 μm [wt]) (cf. Fig. 7B and A) and slightly longer than diploid cells (data not shown). Diploid cells are 1.5× longer (23 ± 0.32 μm) than haploid cells as previously shown (Banuett and Herskowitz 1994). These observations indicate that the *fuz1* gene is required for normal cell length.

***fuz1* mutants exhibit a cell separation defect and secrete a dark pigment**

In the above analysis of cell measurement we observed that *Fuz1⁻* mutant cells exhibit a cell separation defect, which was exacerbated in mid to late log cultures. Large buds remained attached to the mother cell. Both mother and daughter cells continued to bud giving rise to “branched tree” structures (cf. Fig. 7B, C to 7A), indicating that the *fuz1⁻* mutation interferes with normal cell separation. The mother cell budded next to the original site of budding and the daughter budded at its distal end with respect to its site of birth (Fig. 7C), never near its site of birth. The mother was capable of budding multiple times from the junction with the daughter cell (Fig. 7B, C). These observations indicate that *fuz1* is required for cell separation of yeast-like cells. The cell separation phenotype was not suppressed by cAMP (data not shown) as observed for *ubc* mutants (defective in the regulatory subunit of cAMP-dependent protein kinase, which also exhibit a cell separation defect) (Gold et al 1994).

DAPI staining of *fuz1* mutant cells showed a centrally located nucleus throughout most of the cell cycle as in wild type cells (Fig. 7D), which migrates to the bud (data not shown), where it divides, as in wild type cells (Banuett and Herskowitz 2002, Holliday 1965, O'Donnell and MacLaughlin 1984, Steinberg et al 2001). These observations indicate that the *fuz1* gene is not necessary for normal nuclear migration and position in the yeast-like cell.

Cultures of *Fuz1⁻* strains become darkly pigmented after 72 h incubation (Fig. 8C, D), whereas cultures of *Fuz1⁺* strains remained light yellow (Fig. 8A, B), which is the color of the broth. A darkly pigmented supernatant and a whitish pellet are obtained after centrifugation of the culture (data not shown). Microscopic observation indicates that the cell walls are not darkly pigmented. These results indicate that the pigment is secreted and not present in the cell walls. Furthermore, colonies formed by *fuz1* mutants also become darkly pigmented (Fig. 10G). The *fuz1* gene therefore normally inhibits release of a dark pigment by cells in liquid culture and solid agar medium.

***fuz1* mutants exhibit altered septum number and location**

The neck region of *fuz1* mutant cells is ill defined; it is wider than in wild type cells, giving the cells the appearance of long tubes (Fig. 9C). Wild type cells have a narrow neck (Fig. 9A, B) (see Banuett and Herskowitz 2002). These observations indicate that *fuz1* plays a role in constriction of the neck and might suggest a role in cytokinesis or septum formation. We thus viewed septa in asynchronous exponentially growing populations of *fuz1* mutant and wild type cells by staining with Congo red, as described in Banuett and Herskowitz (2002), which stains chitin and other cell wall components (Roncero and Duran 1985), and examined them with epifluorescence microscopy. Large-budded wild type cells contain one or two septa in the neck region between mother and bud, depending on the stage of cell separation (Fig. 9A, B) (see Banuett and Herskowitz 2002). In *fuz1* mutant cells multiple septa locate in the neck region or

its vicinity (Fig. 9C, F, G). Some of the septa are aberrant; in other cases they are mislocalized to the bud (Fig. 9E, H). These observations indicate that the *fuz1* gene is required for proper formation, location and number of septa.

***fuz1* mutants are sensitive to cell wall perturbing agents and to high salt concentration**

Altered colony and cell morphology may reflect defects in cell wall properties. We thus tested growth properties of wild type and *fuz1* mutant strains on media containing Congo red and Calcofluor white, which are known to interfere with cell wall formation (Ram and Klis 2006, Roncero and Duran 1985) (see MATERIALS AND METHODS). Growth of *fuz1*⁻ and *fuz1* null mutant strains was severely inhibited by these compounds suggesting that the mutants have altered cell wall properties or composition (Fig. 10D, E). The strains also exhibit reduced growth on rich medium containing high salt (cf. Fig. 10C and Fig. 10A) (see MATERIALS AND METHODS), indicating that *fuz1*⁻ mutants are sensitive to increased salt concentration in the medium and supports the notion that the mutants have a defective cell wall. Growth of the mutants is reduced slightly on rich medium containing 1M sorbitol (Fig. 10B). The presence of the microtubule depolymerizing drug thiabendazole did not affect growth of the mutant strains to a greater degree than that of wild type strains (data not shown).

The *fuz1* null mutant colonies exhibit sectors of dark- and light-pigmented areas (Fig. 10H). The light sectors are larger in the FB2 than in the FB1 background (Fig. 10H). The original UV-induced mutants exhibit reduced sectoring. These observations suggest that bypass suppressors of the *fuz1* null mutation or suppressors of pigment production arise readily in culture. Alternatively, the strain may exhibit mitotic instability. Analysis of cellular morphology from the light-pigmented and darkly pigmented sectors indicates that cells exhibit the same Fuz1 mutant cell morphology phenotype (data not shown), consistent with the notion that suppressors of pigment production and not bypass suppressors of the deletion arise readily in culture in strains lacking the *fuz1* gene. Sectoring appears to be more extensive in the FB2 than in the FB1 background (cf. Fig. 10He, f and g, with h). The reason for this difference is not known. Strains FB1 and FB2 are sister segregants from a single meiosis (Banuett and Herskowitz 1989), thus they are congeneric not isogenic strains and therefore may carry modifiers responsible for the observed differences.

DISCUSSION

Fuz1 is a member of the MYND domain family of proteins

The putative Fuz1 protein contains a MYND domain, a cysteine-rich, zinc binding domain, highly conserved from yeast to mammalian cells. It is found in proteins involved in embryonic development and cancer (Spadaccini et al 2006). The domain is named after myeloid translocation protein 8 MTG8 (Miyoshi et al 1993), Nervy (Feinstein et al 1995) and DEAF-1 (Gross and McGinnis 1996) and consists of seven cysteine residues and one histidine residue arranged in a C4-C2HC consensus ([CH]-X(2,4)-C-X(7,17)-C-X(0,2)-C-X(4)-[YFT]-C-X(3)-[CH]-X(6,9)-H-X(3,4)-C). The MYND domain has been implicated in protein-protein interactions, in particular in recruitment of partners for proteins involved in transcriptional regulation (Spadaccini et al 2006 and references therein).

Fungal homologues of Fuz1 share a highly conserved hydrophobic amino-terminus domain of unknown function. They also share a highly conserved stretch of amino acids immediately upstream of the MYND domain, which is not present in nonfungal proteins. Whether this region contributes to function of the fungal MYND domain remains to be determined. Little is known of the role of the fungal MYND-domain proteins, except for *Aspergillus nidulans* SamB and *Saccharomyces cerevisiae* Mub1 (Krüger and Fischer 1998) (see below). Our work provides

information on a new member of this group of fungal proteins and supports a role in fungal morphogenesis.

fuz1 is necessary for conjugation tube formation and for maintenance of filamentous growth

Because formation of dikaryotic filaments requires two basic steps, cell fusion and growth of the dikaryon as a filament, mutations that abolish the first step also abolish the second, whereas those that abolish the second do not necessarily affect the first. To distinguish between these possibilities we made use of different assays and different sets of strains. First, using a liquid assay and haploid strains (Banuett and Herskowitz, 1994b), we demonstrated that *fuz1* is required for formation of conjugation tubes; a *fuz1* null mutation blocks this morphological differentiation in coculture of two *fuz1* mutants. Conjugation tube formation occurs in response to pheromones produced by cells of opposite mating type. This response requires different *a* alleles but is independent of the *b* locus (Banuett and Herskowitz 1994a, b; Snetselaar et al 1996, Spellig et al 1994). Thus *fuz1* participates in this *a*-locus dependent pathway. Fuz1 may be a down-stream target of the pheromone response pathway or an upstream regulator that controls expression of one or more components of the pathway.

The weak reaction observed in coculture of wild type and *fuz1* mutants could be due to inability of the *fuz1* mutants to respond to the pheromone produced by the wild type strain. In this scenario the *fuz1* mutant is thought to produce some pheromone that elicits a weak response in the wild type strain. Alternatively, *fuz1* mutants cannot produce pheromone but the pheromone produced by the wild type strain elicits a weak response in the mutant. Future experiments might help distinguish these possibilities.

We made use of diploids that bypass the cell fusion step to examine the role of *fuz1* in postfusion events and demonstrated that *fuz1* is necessary for maintenance of filamentous growth. Filamentous growth of diploids and dikaryons on charcoal agar requires different *a* and *b* alleles (Banuett and Herskowitz 1989, 1994b; Bölker et al 1992, Day et al 1971, Holliday 1961, Puhalla 1968, Ruiz-Herrera et al 1995, Spellig et al 1994). Diploids homozygous at *a* and heterozygous at *b* or homozygous at *b* and heterozygous at *a* are nonmycelial (Banuett and Herskowitz 1989, Day et al 1971, Holliday 1961, Puhalla 1968). Inactivation of both *fuz1* alleles in a diploid heterozygous at *a* and *b* blocks formation of filaments. *fuz1* could function in the *a*-dependent or *b*-dependent pathway of filamentous growth. Because expression of *fuz1* does not appear to increase in the presence of the active bE-bW heterodimer using microarray analysis (M. Vranes and J. Kaämper pers comm), it is likely that *fuz1* is not a component of the *b* pathway. Given that *fuz1* participates in conjugation tube formation, an *a* locus-dependent process, it is most likely that *fuz1* also participates in the *a*-dependent autocrine response regulating filamentous growth. Fuz1 is the first member of the fungal MYND domain family of proteins for which a role in the pheromone response has been demonstrated.

Although *fuz1* mutants do not form filaments on charcoal agar they form filaments in the plant (Banuett and Herskowitz 1996). This observation led to the hypothesis that alternative pathways for filament formation are activated in the plant. Preliminary evidence indicates that in planta the *fuz1* null mutants behave similarly to the UV-induced mutants described by Banuett and Herskowitz (1996). Analysis of the infection process by *fuz1* null mutants will be presented elsewhere.

fuz1 is required for events at the junction of cells

Consistent with a requirement for events at the junction of cells we demonstrated that *fuz1* mutants exhibit a cell separation defect and cells continue to bud while attached to other cells giving rise to tree-like structures. In addition *fuz1* mutants form septa that are aberrant

(incomplete) or mislocalized or contain an increased number of septa in the neck region, near it or in the bud. Last, *fuz1* mutants fail to constrict the neck region. These phenotypes demonstrate that Fuz1 plays a crucial role in events at the boundary of mother and daughter cell (see below). The tree-like structures formed by *fuz1* mutants are somewhat similar to those observed in the *don* mutants (Sandrock et al 2006, Weinzierl et al 2002), however, the attached cells in the *don* mutants have a narrow neck similar to that in wild type cells and a single septum. Don1 appears to be a guanine nucleotide exchange factor for the GTPase UmCdc42, and Don3 appears to be a Ste20-like protein (Sandrock et al 2006, Weinzierl et al 2002).

A. nidulans SamB and *S. cerevisiae* Mub1, two homologues of Fuz1, also exhibit defects at the junction of cells. The *samB* mutation was isolated as an extragenic suppressor of the nuclear migration defect of *apsA* mutants (Krüger and Fischer 1996). SamB mutants exhibit mislocalized septa in germlings, and the morphology of the germlings is altered (Krüger and Fischer 1998). Mub1 mutants have a cell separation defect and initiate bud growth before cytokinesis is complete and form clusters of attached cells (Krüger and Fischer 1998). Taken together these observations indicate that the MYND domain proteins Fuz1, SamB and Mub1 play a crucial role in regulating processes at the junction of cells.

The conclusion that *fuz1* is required for events at the junction of cells is further supported by previous observations of *fuz1* mutants in planta. Formation of teliospores involves a carefully orchestrated sequence of events in which separation (fragmentation) of hyphal cells to produce individual cylindrical cells appears to be a crucial step (Banuett and Herskowitz 1996). In *fuz1* mutants this step is blocked; no teliospores are formed and consequently the life cycle is not completed (Banuett and Herskowitz 1996). We hypothesize that the MYND domain of Fuz1 might mediate interaction with other proteins to control events at the junction of cells during budding and also in filamentous cells during growth in the plant.

Three other proteins, Hgl1 (no matches in the protein databases), Hda1 (histone deacetylase) and Rum1 (similar to human retinoblastoma binding protein 2) are also necessary for teliospore formation; mutants lacking these proteins form tumors devoid of teliospores. *hgl1*, *hda1* and *rum1* mutants appear to block a similar step to that blocked in *fuz1* mutants, but unlike the latter their hyphae do not show aberrant cytoplasmic protrusions (Dürrenberger et al 2001, Quadbeck-Seeger et al 2000, Reichmann et al 2002). It is not clear whether these genes are necessary specifically for the same step controlled by *fuz1* or whether they control events upstream or downstream of *fuz1*. The *rum1* and *hda1* mutations do not confer a cell separation defect in yeast-like cells, and *hgl1* mutants have a slight defect (Dürrenberger et al 2001, Quadbeck-Seeger et al 2000, Reichmann et al 2002).

In addition to its role in events at the junction of cells, *samB* like *fuz1* also is required for sexual development (Krüger and Fischer 1998). *samB* mutants are self-sterile; they form fruit bodies but no viable ascospores; the spores lyse inside the cleistothecia (Krüger and Fischer 1998). Spore lysis might be indicative of altered cell wall properties. In *U. maydis*, *fuz1*⁻ hyphae form aberrant cytoplasmic protrusions suggestive of a weak cell wall, an observation that supports our conclusions above (see RESULTS) that *fuz1* mutants have altered cell wall properties.

Our analysis has shown that the Fuz1 protein is required for cell morphogenesis of the yeast-like cell in *U. maydis* and that it shares some of its roles with its homologues, samB of *A. nidulans* and Mub1 of *S. cerevisiae*.

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Fig. 1.

Filament formation on charcoal agar by wild type and *fuz1* mutant strains. A. Colonies of strain *a2 b2 fuz1⁻* (FBf10-1i) transformed with a cosmid library were replica mated onto a lawn of strain *a1 b1* (FB1) on charcoal agar. *Fuz⁺* colony (arrow) in a background of *Fuz⁻* colonies demonstrates complementation of the *Fuz⁻* phenotype. B. Saturated cultures of strains (a) *a1 b1* (FB1), (b) *a2 b2* (FB2), (c) *a2 b2 fuz1⁻* (FBf10-1i), (d) FB1, (e) and *a2 b2 Δfuz1::hygB* (FB18) were grown in UMC broth overnight and cross-streaked on charcoal agar. The white fuzziness in the cross-streak of (a) and (b) is due to formation of dikaryotic filaments and was recorded 24 h after cross-streaking. C. Saturated cultures of strains (a) *a1/a2 b1/b2* (FBD-12), (b) *a1/a2 b1/b1* (FBD12-3), and three independent *fuz1⁻* diploids, *a1/a2 b1/b2 Δfuz1/fuz1⁻* (c) Df1-1, (d) Df1-2, and (e) DF1-3 were grown in UMC broth overnight, spotted on charcoal agar and photographed after 48 h. The white fuzziness in (a) is due to formation of diploid filaments, which are indistinguishable from dikaryotic filaments.

Fig. 2.

Features of the Fuz1 protein. A. Schematic diagram showing the different domains present in the Fuz1 protein. B = BamHI. The 5kb BamHI fragment is present in p12-F1 (see MATERIALS AND METHODS). B. Position of primers used in the construction of a *fuz1* null mutation and for Southern hybridization and PCR analysis of transformants. X = XhoI sites used in the construction of an internal deletion. M = MscI site used in Southern hybridization analysis.

Fig. 3.

Sequence alignment of Fuz1 and other MYND domain proteins. The amino terminal hydrophobic domain and the MYND domain of Fuz1 were compared to selected proteins from the Basidiomycete fungi, *Cryptococcus neoformans* (gb|EAL19617.1) and *Phanerochaete chrysosporium*; and the Ascomycete fungi, *Neurospora crassa* (gb|EAA31419.1), *Magnaporthe grisea* (gb|EDJ98583.1), *Podospora anserina* (<http://podospora.igmors.u-psud.fr/>), *Aspergillus nidulans* (gb|AJ000996, Krüger and Fischer 1998), *Schizosaccharomyces pombe* (emb|CAB10087.1), and *Saccharomyces cerevisiae* (Z49807). The MYND domain of Fuz1 also was compared to the MYND domain of the three metazoan proteins after which the domain is designated: human MTG8 (X70990, Miyoshi et al 1993), Drosophila Nervy (Feinstein et al 1995), and Drosophila DEAF-1 (Gross and McGinnis 1996). Highlighted amino acids show identities between *U. maydis* Fuz1 and other proteins.

Fig. 4. Southern hybridization analysis of wild type and *fuz1* null mutants. Genomic DNA was digested with *MscI*, fractionated on a 0.7% agarose gel, transferred to a nylon membrane and probed with a 520 bp DIG-labeled 5' UTR fragment in A and reprobbed with a 872 bp DIG-labeled *hygB*-specific fragment in B. Exposure in A and B was for 1 h (see MATERIALS AND METHODS). Lanes in A and B are: (1) = *a1 b1* (FB1); (2) = *a1 b1* Δ *fuz1* (FB21); (3) = *a2 b2* (FB2); (4), (5) and (6) are three independent *a2 b2* Δ *fuz1* strains (FB18, FB19, FB20), respectively; (7) = DIG-labeled molecular weight markers II and VI (Roche).

Fig. 5.

Conjugation tube formation by wild type and *fuz1* mutant strains. Strains were grown and mixed as described in MATERIALS AND METHODS and examined with Nomarski optics. A, I. Pure cultures of strains *a1 b1* (FB1) and *a2 b2 Δfuz1*, respectively, in low nitrogen charcoal liquid medium. B–F. Coculture of strains *a1 b1* (FB1) and *a2 b2* (FB2), 3.5 h (B, C), 8 h (D), and 16 h (E, F) after mixing. Arrowheads in B and C point to developing conjugation tubes. F. DAPI stained filaments 16 h after mixing. g–i. Coculture of strains *a1 b1* (FB1) and *a2 b2 Δfuz1* (FB18), 7.5 h (G), 16 h (H), and 24 h (I) after mixing. Arrowheads in G point to conjugation tubes and arrows in H and I to dikaryotic filaments. Similar results were obtained in coculture of *a1 b1* (FB1) and *a2 b2 fuz1[−]* (FBf10-1i); *a2 b2* (FB2) and *a1 b1 fuz1[−]* (FBf10-3c) (data not shown). J and K. Coculture of strains *a2 b2 Δfuz1* (FB18) and *a1 b1 Δfuz1* (FB21), 7.5 h (J) and 16 h (K) after mixing, respectively. Similar results were obtained in coculture of *a1 b1 fuz1[−]* (FBf10-3c) and *a2 b2 fuz1[−]* (FBf10-1i) (data not shown). Magnification in A–C and G–L is 600×, and in D–F, 400×.

Fig. 6. Colony morphology of wild type and *fuz1* mutants. Strains were streaked on YEPS agar and photographed with a Nikon camera equipped with a macro lens. A and B. FB2 (*a2 b2*) 2-d old colonies; 3 mm diam. C and D. FBf10-1i (*a2 b2 fuz1*⁻) 2-d old colonies, 3 mm diam. Panels A and C demonstrate the uniformity of the colony morphology phenotype in a population. The *fuz1* null mutants exhibit the same colony morphology phenotype.

Fig. 7.

Cell length and cell separation in wild type and *fuz1* mutant cells. Cell length of *fuz1* mutant and wild type cells was determined in asynchronous exponentially growing cultures in YEPS broth and examined with Nomarski optics. Cells also were stained with DAPI and examined with epifluorescence optics to determine nuclear position. A. FB2 (*a2 b2*). B, C and D. FB18 (*a2 b2 Δfuz1*). Panel D. Cells in Panel C stained with DAPI. Arrowheads in C point to emerging buds. Bar = 5 μm.

Fig. 8.

Production of dark pigment by *fuz1* mutants. Strains were grown in YEPS broth and incubated at 28 C overnight and then at RT. A and B. FB2 (*a2 b2*). C and D. FB18 (*a2 b2 Δfuz1*). A and D. 48 h old cultures at RT. B and C. 96 h old cultures at RT.

Fig. 9.

Septum localization and number in wild type and *fuz1* mutant cells. Exponentially growing cultures in YEPS broth were stained with Congo red and examined with epifluorescence optics to view the septa. Strains are *a2 b2* (FB2) in Panels A and B and *a2 b2 Δfuz1* (FB18) in the remaining panels. All *fuz1* mutant cells examined exhibit the same phenotype. A and B. Large-budded wild type cells just before cell separation. The fragmentation zone is the region between the two septa (arrowheads) (Banuett and Herskowitz 2002, McLaughlin and O'Donnell 1984, Weinzierl et al 2002). C, D, F and G. Multiple septa near an ill-defined neck region. Arrowheads in Panel C point to the multiple septa, which are shown at higher magnification in Panel D. E. Multiple septa localized to the bud side of the neck. H. A doublet of closely apposed septa (arrow) in the bud far from the neck region.

FIG. 10.

Growth of wild type and *fuz1* mutant strains on different media. Strains were streaked or spotted on different media (see MATERIALS AND METHODS) and incubated at 28 C. Plates were examined beginning at 72 h and continued 1 wk. In all panels strains are (a) *a1 b1* (FB1), (b) *a2 b2* (FB2), (c) *a2 b2 fuz1*⁻ (FBf10-1i); (d) *a1 b1 fuz1*⁻ (FBf10-3c); e, f, g are three independent *a2 b2* Δ *fuz1* strains (FB18, FB19, FB20, respectively); and (h) is *a1 b1* Δ *fuz1* (FB21). A and F. YEPS agar. B. YEPS 1 M sorbitol. C. YEPS 1 M KCl. D. YEPS Congo red (50 ug/mL). E. YEPS Calcofluor white (40 ug/mL). G and H. YEPS agar showing the same colonies; those in F are 3 d old, and those in G 1 wk. Strains in E and F were streaked on different days than those in A, B, C and D.

Table I*Ustilago maydis* strains used in this study

Strains	Genotype
FB1 ^a	<i>a1 b1</i> prototroph
FB2 ^a	<i>a2 b2</i> prototroph
FB6a ^a	<i>a2 b1</i> prototroph
FB6b ^a	<i>a1 b2</i> prototroph
FBD-12 ^a	<i>a1/a2 b1/b2 ade⁻/ade⁺ met⁻/met⁺</i>
FBD12-3 ^a	<i>a1/a2 b1/b1 ade⁻/ade⁺ met⁻/met⁺</i>
FBf10-11 ^b	<i>a2 b2 fuz1⁻</i>
FBf10-3c ^b	<i>a1 b1 fuz1⁻</i>
FBf10-1c ^b	<i>a1 b2 fuz1⁻</i>
FBf10-5j ^b	<i>a2 b1 fuz1⁻</i>
FBf3c-cbx ^c	FBf10-3c (pCBX122)
FB2-H ^c	FB2 (pCM54)
FBf29 ^c	<i>a1 b1 fuz1ΔXhoI:: hygB</i>
FBf30 ^c	<i>a1 b1 fuz1ΔXhoI:: hygB</i>
FB18, FB19, FB20, ^{cd}	<i>a2 b2 Δfuz1:: hygB</i>
FB21 ^c	<i>a1 b1 Δfuz1:: hygB</i>
FBD12-10 ^c	<i>a1/a2 b1/b2 Δfuz1:: hygB/fuz1⁺</i>
Df1-1, ^{ce}	<i>a1/a2 b1/b2 Δfuz1:: hygB/fuz1⁻</i>

^aBanuett and Herskowitz 1989.^bBanuett 1991.^cThis study.^dFB18, FB19 and FB20 are three independent isolates.^e20 independent diploids were obtained and analyzed.