Aspergillus fumigatus flbB encodes two b-zip proteins required for proper asexual development and gliotoxin production

Peng Xiao\textsuperscript{1,2}, Kwang-Soo Shin\textsuperscript{3}, Tianhong Wang\textsuperscript{1}, and Jae-Hyuk Yu\textsuperscript{2*}

1 State Key Laboratory of Microbial Technology, Shandong University, Jinan, Shandong, P.R. China, 2 Departments of Bacteriology and Genetics, University of Wisconsin, Madison, Wisconsin, 3 Department of Microbiology and Biotechnology, Daejeon University, Daejeon, Republic of Korea

*Corresponding author: Dr. Jae-Hyuk Yu

Address: Department of Bacteriology, 1550 Linden Drive, Madison, WI 53706.

Telephone: (608) 262-4696

Fax: (608) 262-9865

E-mail address: jyu1@wisc.edu

Key words: fungi, Aspergillus fumigatus, development, gliotoxin, transcription factor

Short Title: A. fumigatus developmental regulator FlbB
Abstract

The opportunistic human pathogen *Aspergillus fumigatus* reproduces asexually by forming a massive number of mitospores called conidia. In this study, we characterize the upstream developmental regulator *AfuflbB*. Northern blot and cDNA analyses reveal that *AfuflbB* produces two transcripts predicted to encode two b-zip polypeptides *AfuFlbBβ* (420 aa) and *AfuFlbBα* (390 aa). The deletion of *AfuflbB* results in delayed/reduced sporulation, precocious cell death, the lack of conidiophore development in liquid submerged culture, altered expression of *AfuablA* and *AfuabaA*, and blocked production of gliotoxin. While introduction of the wild type (WT) *AfuflbB* allele fully complemented these defects, disruption of the ATG start codon for either one of the *AfuFlbB* polypeptides leads to a partial complementation, indicating the need of both polypeptides for WT levels of asexual development and gliotoxin biogenesis. Consistent with this, *Aspergillus nidulans flbB* encoding one polypeptide (426 aa) partially complements the *AfuflbB* null mutation. The presence of 0.6 M KCl in liquid submerged culture suppresses the defects caused by the lack of one, but not both, of the *AfuFlbB* polypeptides, suggesting a genetic prerequisite for *AfuFlbB* in *A. fumigatus* development. Finally, Northern blot analyses reveal that both *AfuflbB* and *AfuflbE* are necessary for expression of *AfuflbD*, suggesting that FlbD functions downstream of FlbB/E in aspergilli.
Introduction

The opportunistic human pathogen *Aspergillus fumigatus* (reviewed in 9) propagates in the environment by producing a massive number of mitotic spores called conidia (19). Under appropriate conditions, the conidium germinates and undergoes vegetative growth that leads to the formation of a network of undifferentiated interconnected hyphae known as the mycelium.

After a certain period of hyphal proliferation, in response to appropriate cues, e.g., exposure to air, nutrient deprivation, or osmotic stress, some of the vegetative cells initiate asexual development (conidiation) and go through a series of morphological changes, which results in the formation of conidiophores, consisting of foot cell, stalk, vesicle, phialides and (up to 50,000) conidia (19, 26). Conidia (2 ~ 3 µm in diameter) are then released into the environment and are small enough to reach the alveoli after being inhaled by human (31). In immuno-compromised hosts the conidia are able to germinate into invasive hyphae, which penetrate the vasculature and migrate to distal sites (reviewed in 26).

We have been investigating the mechanisms underlying asexual development and gliotoxin biosynthesis in *A. fumigatus*, primarily focusing on understanding the functions of a number of developmental regulators identified in the model fungus *Aspergillus nidulans* (21, 30, 40). A key step for conidiation in *A. nidulans* (*Ani*) is the activation of *briA* encoding a C2H2 zinc finger transcription factor (TF) (1). Together with *abaA* and *wetA*, these three elements define a central regulatory pathway that coordinates the temporal and spatial control of sporulation-specific gene expression during asexual development in *A. nidulans* (2, 4, 22, 23). Our previous studies have
revealed that the roles of BrlA, AbaA and WetA in developmental regulation are essentially identical in two *Aspergillus* species (20, Tao and Yu, unpublished data).

Identification and characterization of six upstream genes (*fluG*, *flbA*, *flbB*, *flbC*, *flbD*, and *flbE*) required for proper expression of *brlA* in *A. nidulans* have illuminated genetic regulatory cascades leading to the activation of asexual development (2, 23, 33). Among these, *flbB*, *flbC*, *flbD* and *flbE* were defined by the fluffy delayed conidiation mutants (33). FlbB, FlbC and FlbD are putative TFs containing a b-zip, two C2H2 zinc fingers and a cMyb-DNA binding domain, respectively, and are shown to act on the activation of *brlA* expression (10, 11, 14, 16, 32). Recent studies demonstrated that the b-zip TF FlbB is necessary for the expression of *flbD*, and FlbB and FlbD activate *brlA* in a supportive manner (14). The *AnflbE* gene is predicted to encode a 201 aa-length polypeptide with two conserved yet uncharacterized domains, and it was demonstrated that FlbE and FlbB are functionally interdependent, physically interact in vivo, and co-localize at the hyphal tip in *A. nidulans* (13).

Our recent studies have demonstrated that *A. fumigatus* (*Afu*) FlbE is crucial for proper conidiation and is functionally conserved in two aspergilli (17). In this study, we characterize *AfultbB* and present evidence that FlbB is vital for *A. fumigatus* morphological development and gliotoxin production. Unlike *flbB* in *A. nidulans*, *AfultbB* produces two transcripts predicted to encode two b-zip polypeptides *AfuFlbBβ* (420 aa) and *AfuFlbBα* (390 aa). The deletion of *AfultbB* results multiple defects in development, cell viability, and gliotoxin biosynthesis. A series of
complementation studies indicate that both AfuFlbB polypeptides are necessary for proper asexual and chemical development. Supporting this, Aspergillus nidulans f1bB* encoding one polypeptide (426 aa) only partially complements the AfuflbB null mutation. High KCl concentration suppresses the defects caused by the absence of one, but not both, of the AfuFlbB polypeptides, suggesting a genetic prerequisite for AfuFlbB in A. fumigatus conidiation and gliotoxin production. Finally, we show that both AfuflbB and AfuflbE are necessary for expression of AfuflbD and present a model depicting developmental regulation in A. fumigatus.

Materials and methods

Strains, media and culture conditions

Aspergillus strains used in this study are listed in Table 1. Except for the A. fumigatus auxotrophic strains, all fungal strains were routinely grown on solid or liquid glucose minimal medium (MMG) with 0.1% (or 0.5%) yeast extract (YE) at 37°C as previously described (20). For uridine-uracil and arginine auxotrophic mutants (AF293.1 and AF293.6; 34), the medium was supplemented with 5 mM uridine, 10 mM uracil and (for AF293.6) 0.1% arginine. Escherichia coli DH5α was used for routine cloning of constructs and cultured in LB broth, Miller (Novagen, CA) at 37°C supplemented with appropriate antibiotics.

For phenotypic analyses of A. fumigatus strains on air-exposed culture, the conidia (~ 10⁴) of relevant strains were spotted in 2 µl aliquots on appropriate solid medium and incubated at 37°C for the designated time. Conidia were collected in 0.5% Tween 80 from the entire colony and
counted using a hemacytometer. To examine development and secondary metabolite production in liquid submerged culture, spores of relevant strains were inoculated to a final concentration of $10^6$ conidia/ml in 50 ml of liquid MMG with 0.1% YE and incubated at 250 rpm at 37°C for designated time periods. The mycelial aggregates of each strain were observed microscopically starting at 6 h of liquid culture and every 6 h thereafter. To check cell viability in liquid submerged culture, the conidia ($\sim 2 \times 10^5$/ml) of relevant strains were inoculated in 300 ml liquid MMG with 0.1% YE in 1-L flasks and incubated at 37°C at 250 rpm for a designated period. For induction of asexual development and salt stress, the conidia ($\sim 10^6$/ml) of WT and mutant strains were inoculated in 300 ml liquid MMG with 0.1% YE in 1-L flasks and incubated at 37°C at 250 rpm for 18 h (= 0 h for developmental induction). The mycelium was harvested by filtering through Miracloth (Calbiochem, CA), transferred to either solid MMG with 0.1% YE and incubated at 37°C for air-exposed asexual developmental induction, or to liquid MMG with 0.6 M KCl and cultured at 250 rpm, 37°C for salt stress induced development. The plates and mycelia pellets of relevant strains were visually and microscopically examined. Samples collected at various time points of liquid submerged culture and post asexual developmental induction were squeeze-dried, and stored at -80°C, until subject to total RNA isolation.

**Generation of AfuflbB mutants**

The *AfuflbB* gene was deleted in *A. fumigatus* AF293.1 (*pyrG1*) and AF293.6 (*pyrG1; argB1*) strains (34) employing DJ-PCR (37). Oligonucleotides used in this study are listed in Table S1. Briefly, approximately 1.4~1.7 kb of 5' and 3' flanking regions of the *AfuflbB* gene were amplified
from *A. fumigatus* AF293 genomic DNA with the primer pairs oKS31/oKS33 (~1.74 kb) and oKS34/oKS36 (~1.63 kb with the *Anipyrg* tail), and oKS31/oPX76 (~1.43 kb) and oPX77/oKS36 (~1.63 kb with the *AniargB* tail), respectively. The *A. nidulans* selective markers were amplified from FGSC4 genomic DNA with the primer pairs oBS8/oBS9 (*Anipyrg*) and oKH60/oNK105 (*AniargB*). The 5' and 3' flanking regions of *AfuflbB* were fused to each relevant marker and further amplified by the nested primer pairs oKS32/oKS35 and oPX71/oKS35, yielding the final gene deletion constructs. The gene deletion constructs were introduced into recipient strains AF293.1 and AF293.6, respectively. The ∆*AfuflbB* mutants (e.g., TKSS1.01 and TPX2.01) were isolated and confirmed by PCR followed by restricted enzyme digestion (37). At least three deletion strains in each case were isolated.

To complement ∆*AfuflbB*, the wild type (WT) *AfuflbB* allele (ORF with 0.73 kb of 5' and 1 kb of 3' regions) was amplified with the primer pair oPX80/oPX2, digested with *Pvu*II and *Hind*III and cloned into pNJ25 (28). The resulting plasmid pPX1 was then introduced into the recipient ∆*AfuflbB* strain TPX2.01 (∆*AfuflbB*::*AniargB*; *AfupyrG1*), where preferentially a single copy *AfuflbB* gets inserted into the *AfupyrG* locus. Multiple complemented strains were isolated and confirmed by PCR and Northern blot analyses. Similarly, the *AfuflbB* ORF with an 0.73-kb 5' flanking region was amplified using primer pairs oPX80/oPX26, digested and cloned between *Pvu*II and *Hind*III sites in pNJ25 to generate plasmid pPX2, carrying the chimeric *AfuflbB* gene, comprised of the *AfuflbB* native promoter, *AfuflbB* coding region and the *AnitrpC* terminator (35, 36). The resulting plasmid was introduced into the recipient ∆*AfuflbB* strain TPX2.01, and several
strains displaying phenotypes identical to those of \textit{AfuflbB}+ with the native terminator were isolated and confirmed.

To generate the mutants producing only \textit{Afu}FlbBβ or \textit{Afu}FlbBα, the plasmids pPX3 and pPX4 were constructed and introduced into the \textit{ΔAfuflbB} strain TPX2.01. Briefly, the mutagenic primer pairs oPX101/oPX97 and oPX98/102, and oPX101/oPX99 and oPX100/oPX102 were used to amplify different parts of \textit{AfuflbB}, base mismatches are underlined in the joint parts of each amplicon conferring ATG to GCC mutations in each predicted ATG start codon (Table S1). After separate conventional PCR reactions, the amplicons with complementary joint tails were mixed in a 1:1 ratio and subjected to fusion reactions. Then the fused PCR products were amplified using the primer pair oPX80/oPX26 yielding amplicons with 0.73 kb 5’ flanking sequence with the mutated \textit{AfuflbB} ORF: the +1 position ATG or +460 position ATG (Met) was substituted with GCC (Ala). The final PCR products were digested and cloned between \textit{PvuII} and \textit{HindIII} sites in pNJ25 and sequence-verified resulting in pPX3 and pPX4, carrying the chimeric mutated \textit{AfuflbB} gene, comprised of the \textit{AfuflbB} native promoter, \textit{AfuFlbB} coding region, from which only one polypeptide is predicted to be translated, and the \textit{trpC} terminator. Then these two plasmids were introduced into the recipient \textit{ΔAfuflbB} strain TPX2.01, separately. Multiple transformants were isolated and confirmed by PCR. Cross-complemented strains were obtained by introducing a pNJ25 derived plasmid pPX5 carrying \textit{AniflbB}+ (amplified using primer pair oPX90/oPX104 and cloned between \textit{PvuII} and \textit{XbaI} sites in pNJ25) into a \textit{ΔAfuflbB} strain (TPX2.01).
Nucleic acid isolation and manipulation

Genomic DNA and total RNA isolation was carried out as described previously (20, 37). Approximately 10 µg (per lane) of total RNA isolated from individual samples was separated by electrophoresis using an 1.1% agarose gel containing 3% formaldehyde and ethidium bromide, and blotted onto a Hybond-N membrane (Amersham, NY). The $^{32}$P-dCTP labeled hybridization probes for *AfuflbB, AfuflbC, AfuflbD, AfuflbE, AfubrlA, AfuabaA, AfuwetA, AfuvosA* and *gliZ* were prepared by PCR amplification of individual ORFs from the genomic DNA of AF293 by using specific oligonucleotides listed in Table S1. RNA blots were hybridized with individual probes using modified Church buffer (1 mM EDTA, 0.25 M Na$_2$HPO$_4$·7H$_2$O, 1% hydrolyzed casein, 7% sodium dodecyl sulfate; adjusted to pH 7.4 with 85% H$_3$PO$_4$) as previously described (39).

Assessment of cell viability

The Alamar Blue (AB) assay was used to evaluate the cell viability by the percent reduction of Alamar Blue as described (27, 29). Briefly, the amount of alamarBlue cell proliferation indicator (AbD Serotec, NC), equal to 10% of the final volume in the wells, was added into each test well of a 24-well plate (Nunc), containing 1 ml fresh MMG + 0.1% YE and 0.5 ml individual cultures. After 6 h incubation at 37°C, the absorbance of each well was measured at 570 and 600 nm, respectively. The percent reduction of AB was calculated as described previously (29). In addition, to further assess the levels of apoptotic-like cell death, the hyphal cells were stained with Evans Blue as previously described (29). The mycelia of relevant strains grown for certain period were collected and treated with Evans Blue staining solution [1% Evans Blue (Sigma, St. Louis, MO) in
PBS] for 5 min at room temperature, then washed out excessive dye with 10 ml PBS for three times. The samples were analyzed using a bright field microscope.

Gliotoxin analysis

Gliotoxin production was analyzed by thin layer chromatography (TLC) as described (3, 28). To examine gliotoxin production in liquid culture, conidia (~10^6/ml) from individual strains were inoculated into 50 ml liquid MMG with 0.1 % YE in 250 ml flasks and incubated at 37°C, 250 rpm for 48 h. The liquid cultures of designated strains were filtered by Miracloth, 50 ml chloroform was added to the filter-through liquid medium and the mixture was agitated at 250 rpm for 30 min on a rotary shaker at room temperature (28). The aqueous layer was removed, and the chloroform extract was then air dried at room temperature and suspended in 200 µl of methanol. Approximately 15 µl of each sample was loaded onto the silica TLC plates containing a fluorescent indicator (Kiesel gel 60, E. Merck, Germany). Gliotoxin standard was purchased from Sigma (St. Louis, MO). The TLC plates were developed in toluene:ethyl acetate:formic acid (5:4:1, v/v/v). To assess gliotoxin production on solid medium, conidia (~10^4) of designated strains were point-inoculated at the center of MMG with 0.5% YE and cultured at 37°C for 72 h. Equal amounts of agar plugs of the center of colonies were removed and extracted with 15 ml chloroform, agitated vigorously at 250 rpm overnight (3). The chloroform extracts were air dried and suspended in 200 µl of methanol, and then extracts (45 µl/lane) were loaded and analyzed. Photographs of TLC plates were taken following exposure to UV radiation using a Sony DSC-T70 digital camera.
Microscopy

The colony photographs were taken using a Sony DSC-T70 digital camera. Photomicrographs were taken using a Carl Zeiss M² BIO fluorescent microscope installed with a Carl Zeiss AxioVision digital imaging system.

Results

Identification and analyses of *A. fumigatus* FlbB

The putative *A. fumigatus* FlbB gene was identified by a BLASTP search in the *Aspergillus* genome database (Broad Institute *Aspergillus* Comparative Database; http://www.broadinstitute.org/annotation/genome/aspergillus_group/MultiHome.html), with *A. nidulans* FlbB (GenBank accession number: CAM35586.1). Among four matches returned, *Afu2g14680* shows 65% identity, 77% similarity and an expected value of 0. Moreover, a conserved b-zip (pfam00170) domain found in *A. nidulans* FlbB was present at the N terminus (amino acids 65 to 99) of this protein. These results suggest that *Afu2g14680* is likely the *A. fumigatus* homolog of FlbB, and designated as *AfuFlbB*. Additional reciprocal BLASTP searches using the predicted protein sequence of *AfuFlbB* against other *Aspergillus* genomes revealed that the best match is *AniFlbB* in *A. nidulans*, with orthologues of *AfuFlbB* found in other *Aspergillus* species including *A. niger*, *A. oryzae*, *A. clavatus*, *A. terreus* and *A. flavus*, as reported for *AniFlbB* (11). Sequence alignments of FlbB demonstrated that FlbB is highly conserved in
aspergilli; however, the annotated FlbBs in *A. fumigatus* and *A. clavatus* appear to lack 20 ~ 30 amino acids compared to *AniFlbB* (data not shown).

Northern blot analysis of *AfuflbB* mRNA levels throughout the lifecycle of *A. fumigatus* WT (AF293) revealed that the *AfuflbB* gene encodes two distinct transcripts of approximately 2.2 and 1.6 kb, which were designated as *AfuflbBβ* and *AfuflbBα*, respectively (Figure 1A). The levels of the longer transcript are relatively constant throughout the lifecycle, whereas the shorter transcript accumulates specifically along with the progression of asexual sporulation and remains at high levels in conidia. During vegetative growth, the shorter transcript is undetectable and it begins to accumulate at 12 h post asexual developmental induction.

To understand the nature of these two transcripts, various regions of *AfuflbB* cDNAs were amplified from the *A. fumigatus* AF293 Uni-ZAP cDNA library (25; kindly provided by Dr. G.S. May at the University of Texas MD Anderson Cancer Center). Direct sequencing of those PCR amplicons combined with comparison to the genomic sequence of *A. fumigatus* AF293 led to the identification of two overlapping transcription units with identical 3’ ends (Figure 1B). The 2.2 kb *AfuflbBβ* transcription unit contains 4 introns (-220 to -152; +53 to +421; +512 to +574; +1233 to +1282 in Figure 1B) and initiates at -589 with the start codon at +1 resulting in a 420 amino acid polypeptide (accession number HM582656). The 1.6 kb *AfuflbBα* transcription unit contains two introns (+512 to +574; +1233 to +1282 in Figure 1B), initiates at +421 with the first ATG at +460, and is predicted to encode a 390 aa polypeptide, which is essentially identical to *AfuFlbBβ*.
except for the first 30 amino acids at the N terminus (Figure 1C). AfuFlbBα was identical to the predicted protein sequence originally annotated (XP_755800.2). One polypeptide encoded by the AniflbB locus is shown for the comparison (Figure 1C).

The role of AfuFlbB in asexual development

To understand the roles of AfuFlbB in growth and development in A. fumigatus, we first generated \( \Delta \text{AfuflbB} \) strains by replacing the coding region of the AfuflbB genomic locus with the A. nidulans pyrG or argB marker. Complemented strains were subsequently generated by directly integrating the WT AfuflbB allele into the AfupyrG locus of the \( \Delta \text{AfuflbB} \) mutant (TPX2.01). Several \( \Delta \text{AfuflbB} \) strains displaying identical phenotypes were isolated and examined. When point inoculated on solid medium, whereas WT and complemented (C’) strains formed conidiophores abundantly, the \( \Delta \text{AfuflbB} \) mutant exhibited enhanced accumulation of undifferentiated hyphal mass with delayed/reduced conidiation (Figures 2A and S1a). Moreover, the colony edge of WT and C’ strains showed the presence of abundant conidiophores, whereas the \( \Delta \text{AfuflbB} \) mutant exhibited only extended hyphae (Figure 2A middle and bottom panels). Quantitative analyses of conidia per fungal colony grown on solid medium further demonstrated that the asexual spore production in the \( \Delta \text{AfuflbB} \) mutant was dramatically decreased, approximately 50% ~ 70% of WT and C’ strains (Figure S1a). These results suggest that AfuflbB is necessary for normal level conidiation on air-exposed solid culture.

We further examined the effects of \( \Delta \text{AfuflbB} \) during asexual developmental induction. As shown in
Figure 2B, upon induction, the ∆AfuflbB mutant exhibited enhanced proliferation of vegetative cells with delayed progression of conidiation. Moreover, whereas WT strain began to show accumulation of AfubrlA mRNA at 6 h with a peak at 12 h post induction, the ∆AfuflbB mutant started to express AfubrlA at 12 h with a peak at 24 h, i.e., about 6 to 12 h delay in AfubrlA expression. Consequently, AfuabaA expression was delayed in the null mutant as well (Figure 2C). However, expression patterns of AfuwetA and AfuvosA, which are mainly required for the late phase of conidiation, were not different between WT and the null mutant. These results indicate that AfuFlbB is necessary for proper expression of AfubrlA and AfuabaA during the initiation and progression of conidiation.

Effects of ∆AfuFlbB in liquid submerged culture

To further investigate the roles of AfuFlbB, the conidia of WT, ∆AfuflbB and C’ (∆AfuflbB + AfuflbB’) strains were inoculated into liquid MMG with 0.1% YE, and morphologies and cell viabilities were examined. As shown in Figure 3A, whereas WT and C’ strains started to produce simple conidiophores within 18 h, the ∆AfuflbB mutant failed to sporulate even after 96 h in submerged liquid culture (data not shown), indicating that AfuFlbB is required for development in this circumstance. Furthermore, in accordance with these observations, WT accumulated AfubrlA mRNA at 12 ~ 24 h vegetative growth, whereas no AfubrlA expression was detected in the AfuflbB null mutant (see Figure 2C).

To examine whether the absence of AfuflbB influences cell viability, Alamar Blue reduction assays
were carried out (Figure 3B). While all strains examined maintained equally high levels of cell viability until day 3, the \( \Delta \text{AfufibB} \) mutant exhibited drastically reduced cell viability at days 4 and 5. Particularly, at day 4, whereas WT and C' strains showed 94% and 92% of cell viability, respectively, the \( \Delta \text{AfufibB} \) mutant exhibited 52% of cell viability. All strains tested continued to lose cell viability up to day 6 when no differences in the viability were observable (data now shown). To further test whether the reduced cell viability was due to cell death, the hyphal cells of WT, \( \Delta \text{AfufibB} \) and C' strains were stained with Evans Blue staining and microscopically examined. As shown in Figure 3C, compared to WT and C' strains, most of the \( \Delta \text{AfufibB} \) cells were intensely stained with the dye, indicating extensive cell death at day 4. However, when mycelial dry weights of these strains were measured, no obvious differences among WT, C' and \( \Delta \text{AfufibB} \) strains were observable even at days 4 and 5 (data not shown). These results indicate that the absence of \( \text{AfufibB} \) results in accelerated cell death, but not autolysis in liquid submerged culture, and support the idea that fungal autolysis and cell death are separate processes (27).

Both \( \text{AfufibB} \) polypeptides are required for proper asexual development

The presence of two overlapping \( \text{AfufibB} \) transcripts with distinct expression patterns led us to test whether both are required for proper development in \( \text{A. fumigatus} \). This was accomplished by constructing two specific \( \text{AfufibB} \) mutant alleles driven by the native \( \text{AfufibB} \) promoter carrying an ATG (Met) to GCC (Ala) change at each of the predicted start codon (see Figure 4A). Along with the WT allele, each mutant allele was integrated into the \( \text{AfupyrG} \) locus of the \( \text{AfufibB} \) deletion mutant (TPX2.01) and the following three strains were generated (Figure 4A): \( \Delta \text{AfufibB} \ + \text{AfufibB}^+ \)
To test the requirement for the two polypeptides, we first examined the ability of WT, ∆AfulfB (Δ), C’, α and β strains to produce conidia by point inoculating on solid medium. As shown in Figure 4B, whereas the WT AfulfB allele fully complemented ∆AfulfB, the α or β allele restored conidiation to ~80% of WT and C’ strains (Figure S1b), indicating that both polypeptides are required for normal conidiation. To further examine the requirement for two polypeptides, the mycelia of these strains were transferred onto solid medium and progression of conidiation was examined (Figure 4C). As shown, WT and C’ strains started to produce conidiophores at 6 h, whereas the ∆AfulfB mutant began to form conidiophores at 12 h post induction. Similar to ∆AfulfB, AfufBα and AfufBβ strains showed enhanced hyphal proliferation at 6 h and began to sporulate at 12 h post incubation. However, levels of AfubrlA mRNA in these strains were somewhat inconsistent with the delayed conidiation phenotype. As shown in Figure 4C, it appears that α strain started to express AfubrlA from 6 h with a peak at 12 h, followed by a gradual decrease, whereas β strain hardly expressed AfubrlA at 6 h with a peak at 12 h, followed by a dramatic decrease. These results suggest that both polypeptides are necessary for proper asexual development in A. fumigatus and that expression of AfubrlA might be one of many required events for proper conidiation.

The above mentioned strains were further examined in liquid submerged culture for asexual development. As shown in Figure 4D, while WT and C’ strains started to produce conidiophores...
within 18 h, the ∆AfuflbB mutant did not produce any developmental structures at this time and up to 96 h (data not shown). Interestingly, α and β strains failed to produce any conidiophores until 48 h (data not shown), indicating that AfuFlbα and AfuFlbβ are individually required for proper development in liquid submerged culture.

Differential requirement of developmental regulators for proper gliotoxin production

During the cultivation of these strains in liquid submerged culture, we noticed the differences in pigmentation (Figure 5A). We carried out TLC examination of the chloroform extracts of culture filtrates of various strains grown in liquid MMG with 0.1% YE at 37°C for 2 d. As shown in Figure 5B, whereas gliotoxin was clearly detectable in WT and C’ strains, it could not be detected in the chloroform extract of the ∆AfuflbB mutant. On the other hand, a small amount of gliotoxin was detectable in the chloroform extracts of α and β strains. Northern blot analyses indicate that gliZ mRNA (3) is absent in the ∆AfuflbB mutant, but present in WT, C’, α and β strains (Figure 5C). We further examined the ∆AfubrlA and ∆AfubrlA mutants for the ability to produce gliotoxin. These mutants were defective in biosynthesis of gliotoxin in liquid submerged culture (Figure 5D left panel).

However, under air-exposed solid culture condition, WT and mutant strains tested, except for the ∆AfubrlA mutant, produced similar amount of gliotoxin (Figure 5D right panel). These results indicate that AfuflbB is (likely indirectly) associated with gliotoxin biosynthesis and both polypeptides are required for proper production of gliotoxin in liquid submerged culture. Furthermore, the results suggest a potential key role of AfubrlA in gliotoxin biosynthesis (see Discussion).
KCl can suppress the developmental defects caused by the lack of one, but not both, of the AfuFlbB polypeptides

As previous studies demonstrated that unfavorable conditions including salt stress (11) can induce conidia formation in A. nidulans, we investigated whether addition of KCl affects development of the above-mentioned strains. We first tested the effects by point inoculating WT, ∆AfuFlbB, C’, α and β strains on solid MMG containing 0.6 M KCl, and examining the morphologies and conidiation levels. We found that while the ∆AfuFlbB mutant still exhibited delayed/reduced conidiation, WT, C’, α and β strains all formed a normal layer of conidiophores (Figure 6A). Moreover, quantitative analyses of conidia produced by these strains indicate that while the ∆AfuFlbB mutant produced only 70% conidia compared to WT and C’ strains, α and β strains produced the same levels of conidia as WT and C’ strains (data not shown), suggesting that 0.6 M KCl can suppress the developmental defects caused by the absence of one, but not both, of the AfuFlbB polypeptides.

We further examined the suppressive effect of high KCl in liquid submerged culture. These strains were first cultured in liquid MMG with 0.1% YE for 14 h, the mycelium was then collected and transferred into liquid MMG + 0.6 M KCl, and further examined for phenotypic changes. As shown in Figure 6B, WT and C’ strains began to form conidiophores at 12 h post transfer, whereas the ∆AfuFlbB mutant failed to produce any conidiophore-like structures up to 48 h. As observed in solid culture, α and β strains produced conidiophores abundantly just like WT and C’ strains, further suggesting that KCl can replace one, but not both, of the two AfuFlbB
polypeptides for asexual development.

We further investigated whether KCl can suppress the defective gliotoxin production caused by mutations in *AfuflbB*. TLC analyses of the chloroform extracts of the 24 h post KCl transfer cultures (14 + 24 h; Figure 6B) of WT, C', α and β strains showed equal levels of gliotoxin, whereas the ∆*AfuflbB* mutant appeared to be still impaired with gliotoxin production. These results further suggest that KCl can suppress the gliotoxin defect caused by the absence of one, but not both, of polypeptides and that asexual development and gliotoxin production in *A. fumigatus* might be closely associated potentially through *AfuBrlA* (see Discussion).

**AnfFlbB partially complements ∆*AfuflbB***

As *A. nidulans flbB* is predicted to encode only one polypeptide similar to *AfuflbB*, we hypothesized that *AniflbB* cannot fully complement ∆*AfuflbB*. This was tested by introducing *AniflbB* to the *AfupyrG* locus of the ∆*AfuflbB* mutant and examining its conidiation potential. As shown in Figure 7A, when grown on solid medium, ∆*AfuflbB* + *AniflbB* (Ani) strain partially restored asexual sporulation to α and β strains (see Figure 4B for the comparison). Northern blot analysis confirmed that the introduced *AniflbB* gene is highly expressed (Figure 7B). Quantitative analyses of sporulation indicate that *Ani* strain produced conidia to a level similar to that of α and β strains (see Figure S1b). Moreover, similar to α and β strains in liquid submerged culture, *Ani* strain failed to develop up to 48 h (data not shown). In addition, during developmental induction, *Ani* strain exhibited delayed conidiation, despite recovery of near WT level *AfuBrlA* expression, as
observed in α and β strains (Figure 7D). Finally, gliotoxin production was only partially restored by
AniFlbB (Figure 7C). Taken together, these results further corroborate that both AfuFlbB polypeptides are essential for proper asexual development and gliotoxin production, and suggest that A. fumigatus has evolved uniquely with two transcription units of AfuFlbB.

Genetic interactions between upstream developmental regulators in A. fumigatus

To dissect genetic interactions between key developmental regulators, a series of Northern blots were carried out to test the need for AfuFlbB and AfuFlbE for proper expression of AfuflbB-E. As shown in Figure 8A, the absence of AfuflbB or AfuflbE abolished accumulation of AfuflbD mRNA throughout the lifecycle, indicating that both AfuFlbB and AfuFlbE are required for expression of AfuflbD. However, expression of AfuflbB and AfuflbE was not affected by the absence AfuflbE and AfuflbB, respectively. Moreover, expression of AfuflbC was independent of either AfuflbB or AfuflbE. These results suggest that the proposed model for genetic interactions among upstream developmental regulators in A. nidulans (16, 17) is partially applicable for A. fumigatus (see Figure 8B and Discussion).

Discussion

A. fumigatus is an important opportunistic human pathogenic fungus that causes a severe and usually life-threatening systemic mycosis termed “invasive aspergillosis” in immunocompromised individuals (8). In addition to a massive number of small hydrophobic asexual spores, A.
fumigatus produces the toxic secondary metabolite gliotoxin, a key virulence determinant contributing to its pathogenesis (18).

Based on the studies of growth and development in the distantly related A. nidulans, we have been dissecting the roles of genes associated with development in A. fumigatus (17, 20, 40, Tao and Yu, unpublished data). Our previous studies demonstrated that functions of the central regulators BrlA, AbaA and WetA in conidiogenesis are conserved in A. nidulans and A. fumigatus (20, Tao and Yu, unpublished data). On the other hand, while the upstream developmental regulator AfuFlbE plays a crucial role in proper development of A. fumigatus (17), AfuFluG is not essential for conidiation (20). In the present study, we further expanded our understanding of upstream regulation in A. fumigatus development by characterizing AfuflbB. The deletion of AfuflbB resulted in a phenotype similar to that caused by the A. nidulans flbB null mutation, but differing in its severity. In contrast to the ∆AniflbB with a proliferation of undifferentiated aerial hyphae and the near absent conidiophore formation (11), the AfuflbB null mutant exhibited a certain level of asexual development in air-exposed culture conditions although the process was delayed and the conidiation level was reduced. Conversely, AfuflbB deletion strains did not develop in liquid submerged culture, whereas A. fumigatus WT and C' strains elaborated conidiophores abundantly within 24 h. We further asked whether the conidiation defects caused by ∆AfuflbB were associated with altered expression of AfubrlA and other conidiation-specific genes. Consistent with a delay in asexual development in the ∆AfuflbB mutant under synchronized developmental induction conditions (Figure 2B), expression of AfubrlA was delayed.
for 6 to 12 h (Figure 2C). Furthermore, expression of *AfuabaA*, another key regulator directly activated by *AfuBrlA* (Tao and Yu, unpublished data), was delayed about 12 h. However, *AfuwetA* and *AfuvosA*, whose transcripts mainly accumulate at the late phases of asexual development (24, Tao and Yu, unpublished data), were not affected. We speculate two possible explanations for this: one is that *AfuWetA* and *AfuvosA* function in the process of conidium maturation (wall formation and trehalose biogenesis) and confer spore viability, which is a separate phase from the initiation of conidiophore formation activated by *AfuBrlA* and *AfuAbaA* (23); and/or additional components are associated with activation of *AfuwetA* and *AfuvosA* (Tao and Yu, unpublished).

Probably the most important finding of the present study is that two over-lapping mRNAs and polypeptides are encoded by the *AfuflbB* gene, and that both are necessary for proper morphological development and gliotoxin biogenesis in *A. fumigatus*. The longer transcript *AfuflbB*β is present at a relatively constant level throughout the lifecycle whereas the shorter transcript *AfuflbB*α specially accumulates during the progression of conidiation. In *A. nidulans*, only one transcript is encoded by *AniflbB*, which primarily accumulates during asexual development (11). *AniFlbB* (426 aa) shares a high similarity with *AfuFlbB*β (420 aa). Examination of the mutants that produce only one of the two predicted polypeptides reveals that both *AfuFlbB*α and *AfuFlbB*β are required for proper asexual development (Figures 4B and S1b), indicating that the two proteins likely play an additive and/or complementary role. This is supported by the fact that 0.6 M KCl can suppress the developmental defects caused by the absence of one, but not both, of the polypeptides, and that cross-species complementation of
\( \triangle AfuflbB \) with \( AniflbB \) resulted in a partial restoration of asexual development and gliotoxin production as observed in \( AfuflbB\alpha \) and \( AfuflbB\beta \) strains. Our preliminary data indicate that both \( AfuFlbB\beta \) and \( AfuFlbB\alpha \) show the trans-activation ability in yeast (Kwon and Yu, unpublished data), suggesting that both can act as putative b-zip transcription factors. Our Northern blot analyses of the \( \alpha \) and \( \beta \) mutants reveal that only the \( AfuflbB\beta \) mRNA is produced in both \( AfuflbB\alpha \) and \( AfuflbB\beta \) strains, indicating that both \( AfuFlbB\beta \) and \( AfuFlbB\alpha \) are necessary for expression of \( AfuflbB\alpha \), which specifically accumulates during conidiogenesis (data not shown). Additional molecular studies dissecting the complex regulation of the \( AfuflbB \) gene expression need to be carried out.

Aspergillus secondary metabolite production is a complex process, yielding various compounds including carcinogenic mycotoxins such as aflatoxins (AFs) and sterigmatocystin (ST), and is closely associated with morphological development (6, 15, 38). Our present studies suggest that gliotoxin production and asexual development might be inter-connected through the activities of the key developmental regulator BrlA in \( A. fumigatus \). Whereas the deletion of \( AfuflbB \) totally abolished gliotoxin production (Figure 5B) in liquid submerged culture conditions, strains carrying either one or both of \( AfuFlbB\beta \) and \( AfuFlbB\alpha \) produce gliotoxin, of which the amount was reduced compared to WT and C' strains. Indeed, gliotoxin production pattern (Figures 5B & D and 6C) is somewhat consistent with the levels of asexual development (Figures 4B & D, 5A and 6B). This idea was further supported by the fact that under salt stress conditions, where all strains tested except \( \triangle AfuflbB \) formed developmental structures (Figure 6B), \( \alpha \) and \( \beta \) strains restored gliotoxin
production and conidiation to WT level (Figure 6C). Importantly, similar to ∆AfublbB, the absence of AfuflbE abolishes gliotoxin production in liquid submerged culture (Figure 5D) where the ∆AfublbE mutant does not develop (see 17), and ∆AfubrlA eliminates gliotoxin production under liquid and/or solid culture conditions (Figure 5D). These observations suggest that AfuBrI A might play a central role in the cooperative regulation of gliotoxin production and asexual development. Interestingly, multiple BrlA binding sites [BrlA response elements (BREs); 5’-(C/A)(G/A)AGGG(G/A)-3’; 7] are present in the promoter regions of 10 out of the 12 gliotoxin biosynthetic clustered genes (12, 18). It would be of great interest to check whether AfuBrI A indeed binds to these regions and exerts direct regulation (activation) of many of the gliotoxin biosynthetic genes.

Recent studies in A. nidulans demonstrated that FlbB is necessary for expression of flbD encoding a c-Myb protein, and that FlbB and FlbD activate brlA in a cooperative manner (14). Furthermore, it was demonstrated that FlbE and FlbB are functionally interdependent, physically interact in vivo, and co-localized at the hyphal tip in an actin cytoskeleton-dependent manner in A. nidulans and the expression of these two genes are dependent on each other (13, 14). The A. fumigatus flbE gene is predicted to encode a 222 aa-length polypeptide and is required for normal conidiation (17) and gliotoxin production (Figure 5D). Our preliminary study further suggests that AfuFlbD is essential for proper development in A. fumigatus (Xiao and Yu, unpublished data). To examine potential genetic interactions among upstream developmental regulators in A. fumigatus, a series of expression studies were carried out and the results suggest
that *A. fumigatus* possesses an upstream regulatory cascade slightly different from the one proposed in *A. nidulans* (Figure 8A and B). The observations that expression of *AfuFibB* and *AfuFibE* are independent of each other, and that both are required for proper expression of *AfuFibD*, led to a model that *AfuFibB* and *AfuFibE* function upstream of *AfuFibD* and cooperatively activate the expression of *AfuFibD*, which in turn results in activation of *AfuDIA* (Figure 8B).

Although at the transcriptional level *AfuFibB* and *AfuFibE* are independent of each other (Figure 8A), we cannot exclude a possibility that the *AfuFibB* and *AfuFibE* proteins interact and form a functional complex as found in *A. nidulans* (13). In addition, expression of *AfuFibC* is independent of *AfuFibB* and *AfuFibE*, indicating that *AfuFibC* functions in a separate pathway, as found in *A. nidulans*, characterization of *AfuFibC* and additional developmental controllers is in progress.

**Acknowledgments**

We thank Ellin Doyle for reviewing the manuscript. PX was supported by Graduate Scholarship Program sponsored by China Scholarship Council (CSC) and the Ministry of Education of China. The work carried out at Daejon University was supported by Basic Science Research Program through the National Research Foundation of Korea (NRF) funded by the Ministry of Education, Science and Technology (2010-0007836) to KSS. This work was primarily supported by National Science Foundation (IOS-0640067 and IOS-0950850) and Food Research Institute grants to JHY.
References


FlbE in *Aspergillus fumigatus* and *Aspergillus nidulans*, Fungal Genet. Biol. DOI: 10.1016/j.fgb.2010.08.009


Table 1. Aspergillus strains used in this study

<table>
<thead>
<tr>
<th>Strain name</th>
<th>Relevant genotype</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>AF293</td>
<td>A. fumigatus wild type</td>
<td>5</td>
</tr>
<tr>
<td>AF293.1</td>
<td>AfupyrG1</td>
<td>34</td>
</tr>
<tr>
<td>AF293.6</td>
<td>AfupyrG1; AfuargB1</td>
<td>34</td>
</tr>
<tr>
<td>A1176</td>
<td>AfupyrG1; ∆AfubrlA::AfupyrG+</td>
<td>FGSC(^a), 20</td>
</tr>
<tr>
<td>TKSS1.01</td>
<td>AfupyrG1, ∆AfulfB::AnipyrG+</td>
<td>This study</td>
</tr>
<tr>
<td>TPX2.01</td>
<td>AfupyrG1, ∆AfulfB::AfiargB+; AfuargB1</td>
<td>This study</td>
</tr>
<tr>
<td>TPX5.10</td>
<td>AfupyrG1::AfulfB(p)::AfulfB::AfulfB(t)::AfupyrG+, ∆AfulfB::AfiargB+; AfuargB1</td>
<td>This study</td>
</tr>
<tr>
<td>TPX6.01</td>
<td>AfupyrG1::AfulfB(p)::AfulfB::AnitrpC(t)::AfupyrG+, ∆AfulfB::AfiargB+; AfuargB1</td>
<td>This study</td>
</tr>
<tr>
<td>TPX7.06</td>
<td>AfupyrG1::AfulfB(p)::AfulfB::AnitrpC(t)::AfupyrG+, ∆AfulfB::AfiargB+; AfuargB1</td>
<td>This study</td>
</tr>
<tr>
<td>TPX8.02</td>
<td>AfupyrG1::AfulfB(p)::AfulfB::AnitrpC(t)::AfupyrG+, ∆AfulfB::AfiargB+; AfuargB1</td>
<td>This study</td>
</tr>
<tr>
<td>TPX9.03</td>
<td>AfupyrG1::AfulfB(p)::AfulfB::AfulfB(t)::AfupyrG+, ∆AfulfB::AfiargB+; AfuargB1</td>
<td>This study</td>
</tr>
<tr>
<td>TKSS6.07</td>
<td>∆AfulfE::AnipyrG+; AfupyrG1</td>
<td>17</td>
</tr>
<tr>
<td>FGSC4</td>
<td>A. nidulans wild type</td>
<td>FGSC(^a)</td>
</tr>
</tbody>
</table>

\(^a\) Fungal Genetic Stock Center
**Figure legends**

**Figure 1. Summary of *AfuflbB***. (A) Northern blot showing mRNA levels of *AfuflbB* throughout the lifecycle of *A. fumigatus* WT (AF293) strain. Con represents conidia. Numbers indicate the time (hours) of incubation in liquid submerged culture (Veg) and post asexual developmental induction (Asex). Equal loading of total RNA was evaluated by ethidium bromide staining of rRNA. A band shown between the two *AfuflbB* transcripts is due to non-specific binding of the probe to rRNA. (B) Schematic presentation of a genomic DNA region covering the *AfuflbB* gene and two transcripts. The *AfuflbB* ORF (shaded box), transcripts (the arrows) and the introns (shown by discontinuity in the arrow) were verified by sequence analysis of *AfuflbB* cDNA. The start codon A(TG) of *AfuFlbBβ* is assigned as +1. (C) Alignment of N-terminus of FlbB proteins in *A. fumigatus* (*Afu*) and *A. nidulans* (*Ani*). The predicted N-terminus (Met) of *AfuFlbBβ*, *AfuFlbBα* and *AniFlbB* is marked. Note that the 42nd amino acid in *A. nidulans* FlbB is not methionine but isoleucine. Alignment was done by ClustalW (http://www.ch.embnet.org/software/ClustalW.html) and presented by BOXSHADE 3.21 (http://www.ch.embnet.org/software/BOX_form.html).

**Figure 2. Requirement of *AfuflbB* for proper asexual development.** (A) Photographs of the colonies of WT (AF293), *ΔAfuflbB* (TKSS1.01) and complemented (C', TPX5.10) strains grown on solid MMG with 0.5% YE at 37°C for 3 d (top panels) and the close-up views of the colonies (middle and bottom panels). (Bar = 1 cm, top panels; 500 µm, middle and bottom panels) (B) Progression of synchronized asexual development of WT (AF293), *ΔAfuflbB* (Δ, TKSS1.01) strains on solid MMG with 0.1% YE. Numbers indicate the time (hours) post induction. Note the
color differences between WT and ∆AfufibB strains. (bar = 200 µm) (C) Northern blot analyses for levels of AfubriA, AfuabaA, AfuwetA and AfuvosA transcripts in WT (AF293) and ∆AfufibB (TKSS1.01) strains during the lifecycle. Numbers indicate the time (hours) in liquid MMG with 0.1% YE (Veg) or post asexual developmental induction (Asex). Equal loading of total RNA was demonstrated by ethidium bromide staining of rRNA.

**Figure 3. Effects of ∆AfufibB in liquid submerged culture.** (A) Photomicrographs of the mycelium of WT (AF293), ∆AfufibB (∆, TKSS1.01) and complemented (C’, TPX5.10) strains grown in liquid MMG with 0.1% YE for 18 h at 37°C, 250 rpm. The arrows indicate conidiophore structures. Note that WT and C’ strains started to produce vesicles at 18 h, whereas the ∆AfufibB mutant fails to develop. (bar = 50 µm) (B) Viability of WT (AF293), ∆AfufibB (∆, TKSS1.01) and complemented (C’, TPX5.10) strains grown in liquid MMG with 0.1% YE at 37°C, 250 rpm, for the period of 5 days. Data represent the mean values (±SD) of three independent experiments. Note that the ∆AfufibB mutant exhibits accelerated cell death at days 4 and 5. (C) Apoptotic cell death levels of WT (AF293), ∆AfufibB (∆, TKSS1.01) and complemented (C’, TPX5.10) strains grown in liquid MMG with 0.1% YE at 37°C for 4 days were examined by Evans Blue staining. Note the clear differences in the levels of staining among the mycelia (bar = 50 µm).

**Figure 4. Both polypeptides are required for proper asexual development.** (A) Schematics of the three AfufibB alleles used for complementation. The predicted start codons (ATG) of the AfufibB⁺ allele (left panel) were substituted with GCC, respectively, leading to the AfufibBα
(middle panel) and AfuflbBβ (right panel) alleles. (B) Photographs of the colonies of WT (AF293), ∆AfuflbB (Δ, TKSS1.01), C’ (TPX6.01; ∆AfuflbB + AfuflbBα), α, (TPX8.02; ∆AfuflbB + AfuflbBα), and β (TPX7.06; ∆AfuflbB + AfuflbBβ) strains grown on solid MMG with 0.1% YE for 3 d. (bar = 1 cm). (C) Progression of synchronized asexual development of WT (AF293), ∆AfuflbB (Δ, TKSS1.01), C’ (TPX6.01), α (TPX8.02) and β (TPX7.06) strains on solid MMG with 0.1% YE. Numbers indicate the time (hours) post induction. (bar = 200 μm) Right panel shows the corresponding Northern blot of the samples taken from developmental induction. Equal loading of total RNA was demonstrated by ethidium bromide staining of rRNA. (D) Photomicrographs of the mycelium of WT (AF293), ∆AfuflbB (Δ, TKSS1.01), C’ (TPX6.01), α (TPX8.02) and β (TPX7.06) strains grown in liquid MMG with 0.1% YE at 37°C, 250 rpm for 18 h. Note ∆AfuflbB, α, and β strains do not produce any conidiophore structures, whereas WT and C’ strains start to form vesicles (marked by arrows) at 18 h. (bar = 50 μm)

Figure 5. Differential requirement of AfuflbB and other developmental regulators for gliotoxin production. (A) Photographs of relevant strains grown in liquid submerged culture for 3 d. Note the differences in pigmentation. (B) TLC of gliotoxin produced after 2 d liquid submerged culture of WT (AF293), ∆AfuflbB (Δ, TKSS1.01), C’ (TPX6.01), α (TPX8.02) and β (TPX7.06) strains. Toluene:acetate:formic acid (5:4:1) was used as developing solvent. G: gliotoxin standard. (C) Effects of ∆AfuflbB on expression of gliZ. Northern blot of gliZ at 2 d is presented. Equal loading of total RNA was demonstrated by ethidium bromide staining of rRNA. (D) TLC of the chloroform extracts of various strains including the ∆AfuflbE (TKSS6.07) and
ΔAfubrlA (A1176) mutants grown in liquid MMG with 0.1% YE for 2 d or on solid MMG with 0.5% YE for 5 d. Note the lack of gliotoxin production by ΔAfubrlA. G: gliotoxin standard.

**Figure 6. Differential suppression of developmental defects by 0.6 M KCl.** (A) Photographs of the colonies of WT (AF293), ΔAfulfB (Δ, TKSS1.01), C’ (TPX6.01), α (TPX8.02) and β (TPX7.06) strains growth on MMG with 0.1% YE and 0.6 M KCl at 37°C for 3 d. (bar = 1 cm) (B) Photomicrographs of the mycelium of WT (AF293), ΔAfulfB (Δ, TKSS1.01), C’ (TPX6.01), α (TPX8.02) and β (TPX7.06) strains cultured for 14 h in liquid MMG with 0.1% YE then transferred into MMG + 0.6 M KCl. Numbers indicate incubation time post transfer. Conidiophore structures are marked by arrows. Note that only the ΔAfulfB mutant failed to develop. (bar = 50 µm) (C) TLC of gliotoxin in the presence of 0.6 M KCl in liquid submerged culture. After grown in liquid MMG with 0.1% YE for 14 h, the mycelium of relevant strains were transferred into MMG + 0.6 M KCl and further culture at 37°C for 24 h. G: gliotoxin standard.

**Figure 7. Partial complementation of ΔAfulfB by AnilfB.** (A) Phenotypic analyses of WT (AF293), ΔAfulfB (Δ, TKSS1.01), complemented (C’; ΔAfulfB + AfulfB", TPX5.10) and cross-complemented (Ani; ΔAfulfB + AnilfB"; TPX9.03) strains grown on solid MMG with 0.1% YE, 37°C for 3 d (Solid), or in liquid MMG with 0.1% YE, 37°C, 250 rpm, for 18 h (Liquid). (bar = 1 cm, left panels; 50 µm, right panels). (B) Northern blot showing mRNA levels of AfulfB and AnilfB in A. fumigatus WT (AF293), ΔAfulfB (TKSS1.01), C’ (TPX5.10), Ani (TPX9.03) and A. nidulans WT (FGSC4) strains (C: conidia; V: 12 h vegetative; AS: 12 h asexual induction). Note
that AniflbB is highly expressed under the ∆AfuflbB background. (C) TLC for gliotoxin production in liquid submerged culture of relevant strains for 3 d. G: gliotoxin standard. (D) Progression of synchronized asexual development in WT (AF293), ∆AfuflbB (Δ, TKSS1.01), C’ (TPX5.10) and Ani (TPX9.03) strains on solid MMG with 0.1% YE. Numbers indicate the time (hours) post induction. (bar = 200 µm) Right panel shows the corresponding Northern blot for AfubrlA in the samples taken from asexual induction. Equal loading of total RNA was demonstrated by ethidium bromide staining of rRNA.

Figure 8. Genetic interactions between upstream developmental regulators in A. fumigatus.

(A) Levels of AfuflbB, AfuflbC, AfuflbD and AfuflbE transcripts in WT (AF293), ∆AfuflbB (TKSS1.01) and ∆AfuflbE (TKSS6.07) strains throughout the lifecycle. Numbers indicate the time (hours) in liquid MMG with 0.1% YE (Veg) or post asexual developmental induction (Asex). A band shown between the two AfuflbB transcripts is due to non-specific binding of the probe to rRNA. (B) A genetic model for upstream regulation of asexual development in A. fumigatus (see Discussion).
Figure 1
Fig 2
Figure 3
Figure 4
Figure 5
Figure 6

A

WT  ΔAfuflbB  C'  α  β

B

Post transfer to MMG + 0.6 M KCl

6  12  24 h

WT

Δ

C'

α

β

C

MMG + 0.6 M KCl

(14 + 24 h)

G  WT  Δ  C'  α  β  G

Figure 6
Figure 7
Figure 8