Gβ-Like CpcB Plays a Crucial Role for Growth and Development of Aspergillus nidulans and Aspergillus fumigatus

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Abstract
Growth, development, virulence and secondary metabolism in fungi are governed by heterotrimeric G proteins (G proteins). A Gβ-like protein called Gib2 has been shown to function as an atypical Gβ in Gα1-cAMP signaling in Cryptococcus neoformans. We found that the previously reported CpcB (cross pathway control B) protein is the ortholog of Gib2 in Aspergillus nidulans and Aspergillus fumigatus. In this report, we further characterize the roles of CpcB in governing growth, development and toxigenesis in the two aspergilli. The deletion of cpcB results in severely impaired cellular growth, delayed spore germination, and defective asexual sporulation (conidiation) in both aspergilli. Moreover, CpcB is necessary for proper expression of the key developmental activator brlA during initiation and progression of conidiation in A. nidulans and A. fumigatus. Somewhat in accordance with the previous study, the absence of cpcB results in the formation of fewer, but not micro-, cleistothecia in A. nidulans in the presence of wild type veA, an essential activator of sexual development. However, the cpcB deletion mutant cleistothecia contain no ascospores, validating that CpcB is required for progression and completion of sexual fruiting including ascosporesogenesis. Furthermore, unlike the canonical GβSfaD, CpcB is not needed for the biosynthesis of the mycotoxin sterigmatocystin (ST) as the cpcB null mutant produced reduced amount of ST with unaltered STC gene expression. However, in A. fumigatus, the deletion of cpcB results in the blockage of gliotoxin (GT) production. Further genetic analyses in A. nidulans indicate that CpcB may play a central role in vegetative growth, which might be independent of FadA- and GanB-mediated signaling. A speculative model summarizing the roles of CpcB in conjunction with SfaD in A. nidulans is presented.

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Introduction
Components of a heterotrimeric G protein (G protein) including FadA (Gα), GanB (Gβ), SfaD (Gβ) and GpgA (Gγ) govern spore germination, vegetative growth, development, stress response, and toxigenesis in the model filamentous ascomycete Aspergillus nidulans [1]. Genetic studies have revealed that activated FadA-GTP transduces signals in part through a cAMP-dependent protein kinase (PkaA), leading to stimulation of vegetative proliferation, inhibition of sexual and asexual development, and suppression of biosynthesis of the carcinogenic mycotoxin sterigmatocystin (ST), the penultimate precursor of the better-known aflatoxins [2–4]. FlbA is an RGS (regulator of G protein signaling) protein that negatively regulates FadA-mediated growth signaling likely by enhancing the intrinsic GTPase activity of FadA [5,6]. Loss of flbA function and FadA dominant-activating mutations cause uncontrolled proliferation of undifferentiated vegetative cell mass followed by colony autoysis. The null or dominant interfering mutant alleles of fadA bypass the need for FlbA in conidiation and ST production [2,7]. RgsA is a specific RGS protein that primarily attenuates GanB signaling [8]. GanB-mediated signaling stimulates spore germination and stress responses, and inhibits asexual sporulation (conidiation) in part through PkaA. The rgsA deletion mutant exhibited a phenotype highly similar to that of the GanBΔ (Q208L) mutant [9,10], i.e. reduced colony size, elevated germination without external carbon sources and accumulation of dark brown pigments. Conversely, the deletion of ganB suppresses the phenotypes caused by ΔrgsA [8].

The A. nidulans Gβγ subunits play an equally important role in governing growth and development. The canonical Gβ subunit SfaD is composed of 352 amino acids sharing ~60% identity with mammalian Gβ, and has a conserved Trp-Asp sequence referred to as the “WD-40” motif [11]. The single Gγ subunit GpgA consists of 90 amino acids showing 72% similarity with the yeast Ste18p [12]. GpgA contains a typical coiled-coil domain at the N-terminal region, which is necessary for the interaction of a Gγ with...
a cognate Gβ to form a heterodimer [13]. The deletion of sfad or gpgA results in highly restricted vegetative growth and rescues certain conidiation defects caused by the absence of flkB, providing a key genetic evidence that SfAD and GpgA function in the FadA-mediated vegetative growth signalling. The SfAD·GpgA heterodimer also functions with GanB, and GanB and SfAD·GpgA are associated with spore germination, carbon source sensing, stress responses and hyphal pigmentation in A. nidulans [11,13,14]. Because the deletion of either sfad or gpgA results in the lack of chroistothecia (sexual fruiting bodies) in self-fertilization and causes a severe impairment in sexual development in outcrosses, it has been proposed that SfAD and GpgA constitute the primary signaling component for sexual reproduction in A. nidulans [1,11,13,14].

The opportunistic human pathogen Aspergillus fumigatus causes mycosis, allergy, and invasive aspergillosis in immune-compromised individuals [15]. As G protein components are highly conserved in eukaryotes, the corresponding A. fumigatus orthologs of the above mentioned A. nidulans G protein components show high levels of identity [16–18]. In our previous study, we demonstrated that the FadA homologue GpaA mediates signaling for vegetative growth, which in turn negatively controls conidiation, and that GpaA signaling is attenuated by AfsFlbA [1,18–20]. We also characterized the homologues of SfAD and GpgA in A. fumigatus and presented a series of evidence that AfsFlbA and AfsGpgA play a crucial role in governing vegetative growth, spore germination, conidiation and production of certain secondary metabolites [21].

A novel Gβ-like protein, Gib2, has been identified in the human pathogenic fungus Cryptococcus neoformans as an interacting protein of the Gζ subunit Gpa1 in a yeast two-hybrid screen [22]. Gib2 is a homolog of the Gβ-like/receptor for activated protein kinase C1 (RACK1), which had previously been identified as a putative intracellular receptor for activated protein kinase C [23]. C. neoformans Gib2 contains a seven WD-40 repeat motif and is predicted to form a seven-bladed β propeller structure that is characteristic of β transducins. It was further demonstrated that Gib2 functions as an atypical Gβ in Gpa1-cAMP signaling that likely stabilizes Gpa1 and facilitates its oscillation between ON and OFF status, and thereby regulates cAMP signaling. Gib2 was shown to physically interact with the two Gγ subunits Gpg1 and Gpg2, similar to the conventional Gβ subunit Gpb1. Knock-down of gib2 by antisense suppression resulted in a severe growth defect, indicating that Gib2 plays a key role in promoting vegetative proliferation in C. neoformans [22]. These studies suggest that a Gib2 ortholog might play an important role in Aspergillus proliferation, where three Gζ subunits but only one Gβ subunit has been identified.

Our search for a potential ortholog of Gib2 has identified CpcB in the A. nidulans genome. CpcB was previously identified and characterized as a component of “cross pathway control” [24]. Amino acid limitation results in impaired sexual fruiting in A. nidulans. Hoffman et al [24] revealed that the c-Jun homolog CpcA and the RACK1 homolog CpcB coordinate sexual developmental progression illuminating a possible connection point between metabolism and sexual development. Amino acid starvation activates the cross-pathway regulatory network including the transcriptional activator CpcA, leading to induction of a number of amino acid biosynthetic genes. CpcB negatively regulates the cross-pathway network in the presence of amino acids. When A. nidulans is grown under amino acid starvation conditions, the fungus is unable to complete meiosis and sexual fruiting, producing microcleistothecia filled with hyphae. Addition of amino acids removes this specific developmental block and leads to completing sexual fruiting including the development of mature ascospores. Hoffman et al [24] demonstrated that the identical developmental block is induced by either overexpression of a c-Jun homolog (cpcA, GCN-4, or cpc-1) or deletion of cpcB in the presence of amino acids. Furthermore, the deletion of cpcB resulted in the increased expression of amino acid biosynthetic gene (argB). Collectively, CpcA (activated by aa starvation) inhibits the progression of sexual fruiting before meiosis, and CpcB (represses aa biosynthesis in the presence of aa) confers the progression and completion of sexual development including maturation of ascospores.

In Aspergillus, asexual development (conidiation) results in the formation of a massive number of conidia that form on the specialized structures called conidiophores [25–27]. A key and essential step for conidiophore development is activation of brlA, which encodes a C2-H2 zinc finger transcription factor activating conidiation specific genes and abaA [28,29]. The abaA gene encodes a developmental regulator that is activated during the middle stages of conidiophore development after metulae differentiation [30,31]. The abaA gene functions in late phase of conidiation for the synthesis of crucial cell wall components [32,33]. These three genes have been proposed to define a central regulatory pathway that acts in concert with other genes to control conidiation-specific gene expression and determine the order of gene activation during conidiophore development and spore maturation [25,26,34]. VosA is a velvt-domain protein that exerts feedback regulation of brlA and couples sporogenesis and trehalose biogenesis in spores, required for the long-term viability of conidia and ascospores, thereby completing conidiogenesis [35].

While CpcB's functions in cross-pathway control and sexual fruiting have been characterized, its role in governing spore germination, vegetative growth, and sexual development (conidiation) in A. fumigatus has remained to be investigated. Moreover, Hoffman et al. [24] examined the role of CpcB in sexual development using a lab strain with the vea1 allele [36], which lacks the full potential for sexual development. VeA is the founding member of the fungi-specific velvt proteins, and it forms the VelB-VeC and/or VelB-VeA-LaCA complexes that control the initiation and progression of sexual fruiting and secondary metabolism [36–39]. Therefore, in order to further assess the precise role of cpcB in sexual development, we have characterized the cpcB null mutation with the wild type (WT) vea allele. Here, we report the roles of CpcB in governing growth, asexual/sexual development and toxigenesis in both A. nidulans and A. fumigatus.

Materials and Methods

Aspergillus Strains and Culture Conditions

Aspergillus strains used in this study are listed in Table 1. Fungal strains were grown on solid or liquid minimal glucose medium with appropriate supplements (e.g. 1 g uracil l⁻¹ + 1 g uridine l⁻¹, or 1 mL 1% pyridoxine per liter; simplified as MM), or with yeast extract (YE) as described [40], and incubated at 37°C. Escherichia coli DH5α was used for routine cloning of constructs and cultured in LB broth, Miller (Novagen, CA), at 37°C and supplemented with appropriate antibiotics [41].

For phenotypic analyses of Aspergillus strains on air-exposed culture, conidia (~10⁵) of relevant strains were spotted in 2-μL aliquots on appropriate solid MM and incubated at 37°C for 4 days. Conidia were collected in 0.1% 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 10⁶ conidia/ml in 100 mL of liquid MM,
Table 1. Aspergillus strains used in this study.

| Strain   | Genotype                | Source         |
|----------|-------------------------|----------------|
| FGSC4    | Wild Type (veA⁺)         | FGSC*          |
| RJMP 1.59| pyrG89; pyroA4; veA⁺     | [60]           |
| TNJ36    | pyrG89; pyroA4; AfupyrG⁺; veA⁺ (control strain) | [61]          |
| RJMP1.59-8b | pyrG89, pyroA4; ΔcpcB:AfupyrG⁺; veA⁺  | This study    |
| RJMP1.59-9b | pyrG89, pyroA4; ΔcpcB:AfupyrG⁺; AnicpcB[p];AnicpcB⁺; veA⁺ | This study    |
| TNJ36.1  | pyrG89; pyroA4; pyG⁺; veA⁺ | This study    |
| QK1b     | pyrG89; pyroA4; pyG⁺; ΔflbA-AnipyraA⁺; veA⁺ | This study    |
| QK2b     | pyrG89, pyroA4; pyG⁺; ΔgsA-AnipyraA⁺; veA⁺ | This study    |
| QK3b     | pyrG89, ΔcpcB:AfupyrG⁺; pyroA4, ΔflbA-AnipyraA⁺; veA⁺ | This study    |
| QK4b     | pyrG89, ΔcpcB:AfupyrG⁺; pyroA4, ΔgsA-AnipyraA⁺; veA⁺ | This study    |
| A. fumigatus | AfupyrG1                    | FGSC*          |
| AI293    | Wild Type                | [62]           |
| AI293.1  | AfupyrG1                 | [63]           |
| AI293.1-7b | AfupyrG1; ΔcpcB:AnipyraG⁺ | This study    |
| AI293.1-7C | AfupyrG1; ΔcpcB:AnipyraG⁺; AfucpcB[p];AfucpcB⁺ | This study    |

*Fungal Genetics Stock Center.

1Multiple isogenic strains (all behaved identically).

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or with YE and incubated at 220 rpm at 37°C. For induction of asexual (and sexual) development, the conidia (10⁶/ml) of WT and mutant strains were inoculated in 300 ml of liquid MM, or with YE in 1-liter flasks and incubated at 37°C, 220 rpm for 14 to 18 h (=0-h time point for developmental induction). The mycelium was harvested by filtering through Miracloth (Calbiochem, CA) and transferred to solid MM and incubated at 37°C. The plates and mycelium pellets of relevant strains were visually and microscopically examined. Samples collected at various time points of the liquid submerged culture and post-asexual developmental induction were placed in-between the paper-towel, squeezed to remove excess medium, transferred to a micro-centrifuge tube and stored at -80°C until subjected to total RNA isolation. For the determination of germination rates and the length of hyphae, about 10⁶ conidia of relevant strains was harvested by filtering through Miracloth (Calbiochem, CA) and transferred to solid MM and incubated at 37°C. The plates and mycelium pellets of relevant strains were visually and microscopically examined. Samples collected at various time points of the liquid submerged culture and post-asexual developmental induction were placed in-between the paper-towel, squeezed to remove excess medium, transferred to a micro-centrifuge tube and stored at -80°C until subjected to total RNA isolation. For the determination of germination rates and the length of hyphae, about 10⁶ conidia of relevant strains were spread onto solid MM. Cultures were grown at 37°C, and the spores were monitored at each time point [10].

Construction of A. nidulans Strains

The oligonucleotides used in this study are listed in Table S1. For the deletion of cpcB, double-joint PCR (DJ-PCR) method was used [43]. Both flanking regions of cpcB were amplified using the primer pairs oPX-105/oPX-106 and oPX-107/oPX-108 and using FGSC4 genomic DNA as a template. The A. fumigatus pyrG marker was PCR-amplified from AF293 genomic DNA with the primer pair oJH-83/oJH-86. The three fragments were fused, and the final PCR product for the cpcB deletion was amplified using the primer pair oPX-109/oPX-110, and introduced into RJMP 1.59

protoplasts generated by the Vinollow FCE lysing enzyme (Novo Nordisk) [44]. The ΔafupcB mutants (RJMP1.59-8) were isolated, and confirmed by PCR and subsequent restriction enzyme digestion of the amplicon [43]. At least three independent deletion strains were isolated. Likewise, 5' and 3' flanking regions of flbA (oNK-412/oNK-413 and oNK-414/oNK-415 andrgsA (oNK-540/oNK-541 and oNK-542/oNK-543) were amplified, fused with the A. nidulans pyrG (amplified with oNK-395/oNK-396) selective marker by DJ-PCR, and used to make the final PCR amplicons (oNK-416/oNK-417 and oNK-544/oNK-545). Each construct for the deletion of flbA or rgsA was introduced into TNJ36.1 to acquire the ΔanilflbA (QK1) and ΔanirgsA (QK2) mutants, or introduced into RJMP1.59-8 to acquire the double deletion mutants (QK3 and QK4).

To complement ΔafupcB, the WT AfupcB gene (3673bp) was amplified from FGSC4 by oLW-1 and oLW-2. The KmR and EcoRI digested AfupcB amplicon was cloned into pH8 and the resulting recombinant DNA was introduced into E. coli. Individual clones were sequence verified the final plasmid pLW1 was used to transform RJMP1.59-8 into the complemented strain RJMP1.59-8C, verified by oHS-350 and oHS-351.

Construction of A. fumigatus Strains

The AfupcB gene was deleted in the A. fumigatus AF293.1 [pyrG1] strain employing DJ-PCR [43]. The 5' and 3' flanking regions of the AfupcB gene was amplified from A. fumigatus AF293 genomic DNA with the primer pairsoPX-111/oPX-112 and oPX-113/oPX-114. The A. nidulans pyrG⁺ marker was amplified from FGSC4 genomic DNA with the primer pair oBS-08/oBS-09. The 5' and 3' flanking regions of AfupcB were fused to the marker and further amplified by the nested primer pair oPX-115/oPX-116, yielding the final AfupcB gene deletion construct. The gene deletion construct was introduced into the recipient strain AF293.1, and the ΔafupcB mutants (e.g., AF293.1-7) were isolated and confirmed by PCR followed by restriction enzyme digestion [43]. At least three independent deletion strains were isolated.
To complement Δ\textit{AfupcB}, the hygromycin resistance gene from plasmid pUCH2-8 and the \textit{AfupcB} gene from wild-type AF293 strain were amplified by primer pairs oLW-3/oLW-9 and oLW-10/oPX-114, respectively. Fusing them by single joint PCR with the primer pair oLW-4/oLW-7 resulted in the final 5.8 kb complementation construct, which was then used to transform \textit{Afu}293.1-7C (Δ\textit{AfupcB}) and transformants resistant to 100 μg/ml hygromycin were isolated. The primer pair oLW-3/oLW-11 was used to check the presence of the WT cpeB allele in the transformants, leading to isolation of the complemented strain AF293.1-7C.

Nucleic Acid Isolation and Manipulation

Genomic DNA isolation, total RNA isolation and Northern blot analyses were carried out as described previously [19,43,45]. Total RNA was isolated from conidia and mycelia collected at various time points. All samples were squeeze-dried, quick-frozen in liquid N2 and stored at −80°C until subjected to RNA isolation. Approximately 10 μg (per lane) of total RNA isolated from individual samples was separated by electrophoresis using a 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 \textit{AnivosA}, \textit{AnisU}, \textit{AnictcU}, \textit{AniaflR}, \textit{AnicpcB}, \textit{AnifabaA} and \textit{AfutpyG} were prepared by PCR amplification of individual ORFs from the genomic DNA of FGSC4 and AF293 by using specific oligonucleotides (Table S1; Fig. S1).

ST and GT Analysis

Spores (≈10⁶) of each strain were inoculated into 3 ml liquid MM with 0.5% YE in 8-ml tubes and the stationary cultures were incubated at 37°C for 1, 2, 4, 6, 8 days, respectively, as described [46]. ST was extracted from 1, 2, 4, 6, 8-day-old cultures by adding 1 ml of CHCl₃ to each tube and then vortexing for 1 min. The organic phase was transferred to 1.5 ml tubes and centrifuged at 5000 g for 5 min at 4°C. The CHCl₃ layer was collected, dried, and resuspended in 50 μl of CHCl₃ and 10 μl of each sample was applied onto a thin-layer chromatography (TLC) silica plate containing a fluorescence indicator (Kiesel gel 60, 20 cm×20 cm, 0.25 mm thick; E. Merck). ST standard was purchased from Sigma and about 5 μg was applied onto the TLC plate with the samples. The plate was then developed with toluene:ethylacetate:acetic acid (80:10:10, v/v/v), where the Rᵢ value of ST is about 0.65. At this step ST exhibits dark-red color under the long wave UV (320 nm). To enhance visibility and the detection limit of ST, aluminum chloride (20% AlCl₃·6H₂O in 95% ethanol) is sprayed on to the TLC plate and the plate is baked at 80°C for 5 min. The color of ST changes from red to bright light-green by this process [47]. Photographs of TLC plates were taken following exposure to UV radiation using a SONY DSC-F828 digital camera.

To assess the production of GT, conidia of each strain were inoculated into 50 ml liquid YM and incubated for 2 days at 37°C and 250 rpm. GT was extracted with chloroform as described previously [48]. The chloroform extracts were air-dried and resuspended in 100 μl of methanol. Ten μl of each sample was applied to a thin-layer chromatography (TLC) silica plate containing a fluorescence indicator (Kiesel gel 60, E. Merck). TLC plate was developed with chloroform:acetone (7:3, v/v) until the solvent front reached about 15 cm. GT standard was purchased from Sigma.

Microscopy

Photomicrographs were taken by using a Zeiss Axioplan 2 microscope with AxioVision digital imaging software (Zeiss). Culture plate and Northern blot photographs were taken using a SONY DSC-F828 digital camera.

Statistical Analysis

All experiments were performed triplicates for each condition analyzed. Data are shown as mean±SD and bars indicate SD. Statistical differences between WT and mutant strains were evaluated with Student’s unpaired t-test. P values <0.05 were considered significant.

Results

Gene Structure and Expression of cpeB

BLAST searches of the \textit{A. nidulans} and \textit{A. fumigatus} genome (AspGD) employing the \textit{C. neoformans} Gib2 protein [22] led to the identification of the \textit{cpeB} gene in \textit{A. nidulans} [24] and \textit{A. fumigatus}. The \textit{cpeB} gene maps to chromosome II in \textit{A. nidulans} and the ORF consists of 1,430 bp, interrupted by three introns (144 bp, 128 bp and 213 bp), and is predicted to encode a 316 aa-length protein (AN14163; AF176775). The \textit{A. fumigatus} \textit{cpeB} gene maps to chromosome IV, and its ORF consists of 1447 bp, interrupted by three introns (142 bp, 125 bp and 235 bp), and is predicted to encode a 316 aa-length protein (Afu4g13170). \textit{cpeB} mRNA accumulates at varying levels throughout the lifecycle in both species. In \textit{A. nidulans}, levels of \textit{cpeB} mRNA are detectable at 6 h of vegetative growth, maintained at high levels during the early phases (0–6 h) of asexual development, reduced in the late phase (72–120 h) of conidiation, and absent in conidia (Fig. 1A). In \textit{A. fumigatus}, \textit{cpeB} mRNA is detectable at 6 h of vegetative growth, maintained at high levels throughout the lifecycle in both species.

Requirement of CpcB for Proper Growth and Conidiation

We generated individual null mutants lacking \textit{AnicpcB} (Δ\textit{AnicpcB}; RJP1P-1.5-9-8) and \textit{AfupcB} (Δ\textit{AfupcB}; NA293.1-7-7) by replacing its coding region with \textit{AfupcB}G' and \textit{AnicpcB}G', respectively (see Table 1). Subsequently, using each deletion strain as a transformation host, we further generated complemented strains (C') by introducing the WT \textit{AnicpcB} allele into the \textit{pyrA} locus, and the WT \textit{AfupcB} allele linked with HygR, respectively. We then examined the phenotypes of WT, Δ\textit{cpeB} and complemented strains (C'; Table 1). Most markedly, as shown in Fig. 2A, the deletion of \textit{cpeB} resulted in highly restricted vegetative growth with nearly absent conidiation. Quantitative analyses of conidia per colony grown on solid medium further demonstrated that asexual spore production in the Δ\textit{cpeB} mutant was significantly reduced (p<0.01); approximately 1% of WT and C' strains of \textit{A. nidulans} and ~30% of WT and C' strains of \textit{A. fumigatus} (Fig. 2B). These results suggest that \textit{cpeB} is necessary for proper proliferation and asexual development in air-exposed solid cultures of \textit{A. nidulans} and \textit{A. fumigatus}.

We further examined the effects of Δ\textit{cpeB} during the progression of conidiation upon developmental induction. To minimize the effects of growth defects on expression of \textit{brlA}, the same amount of wet mycelia of WT and the mutants was transferred onto the solid medium, and the equal amount of the developing cells was subject to RNA analyses. During vegetative growth of \textit{A. nidulans}, no differences in mRNA levels of \textit{brlA}, \textit{abaA}, \textit{wetA} and \textit{vocA} between...
WT and the null mutant were observable (data not shown). Upon induction of asexual development, however, WT strain began to accumulate $brlA$ mRNA at 6 h, with a peak at 12 h postinduction, whereas the $\Delta cpcB$ mutant started to express $brlA$ at 12 h, with a peak at 48 h, i.e., 12-36 h delay (Fig. 2C). Likewise, WT strain began accumulating $abaA$, $wetA$ and $vosA$ mRNA at 12 h, with a peak at 12-24 h post-induction, whereas the $\Delta cpcB$ mutant exhibited highly reduced and delayed accumulation of $abaA$, $wetA$ and $vosA$ transcripts during the progression of conidiation (see Fig. S1). These results indicate that CpcB is necessary for proper expression of $brlA$, $abaA$, $wetA$ and $vosA$ during the initiation and progression of conidiation in $A. nidulans$.

In $A. fumigatus$, upon induction of asexual development, WT strain accumulated $AfubrlA$ mRNA at 12 h, with a peak at 24 h post-induction, whereas the $\Delta afucpcB$ mutant started to accumulate $AfubrlA$ at 24 h, with a peak at 36 h (Fig. 2C). Similarly, WT strain began accumulating $AfubrlA$ mRNA at 24 h, while the $\Delta afucpcB$ mutant started to express $AfubrlA$ at 36 h; i.e., about a 12 h delay in $AfubrlA$ and $AfubraA$ expression (data not shown). These results indicate that AfuCpcB is necessary for proper expression of $AfubrlA$ and $AfubraA$ during the initiation and progression of conidiation in $A. fumigatus$, too.

CpcB is Necessary for Sexual Development in $A. nidulans$

We further investigated the roles of CpcB in sexual fruiting of $A. nidulans$ in the presence of the WT veA allele. When point-inoculated on MMG, the $\Delta cpcB$ mutant formed smaller, white colonies producing few cleistothecia (Fig. 3A), whereas WT and C’ strains formed cleistothecia abundantly. After incubating on MMG for 7 days, WT and C’ strains produced 800 ± 117.85 and 394 ± 24.58 cleistothecia/cm², respectively. However, the $\Delta cpcB$ mutant produced 23.67 ± 4.73 cleistothecia/cm² and most...
of cleistothecia were small, fragile and with reduced pigmentation (Fig. 3B and 3C). Furthermore, whereas WT and C’ cleistothecia contained 8.66(6 1.82) × 10^4 and 5.7(6 3.27) × 10^4 ascospores per cleistothecium, respectively, the DcpcB cleistothecia contained no ascospores. These results confirm that CpcB is required for proper formation of fruiting bodies and ascosporogenesis [24]. We noted that the presence of the WT cpcB allele in the pyroA locus appears to be insufficient to fully restore sexual fruiting of the DcpcB mutant to WT level. This might be due to the positional effects and/or the lack of key cis acting elements in the cloned cpcB WT gene region in the complementation construct, leading to improper expression of cpcB in trans.

We then examined mRNA levels of vexA and vexD during the progression of sexual development employing homogeneous developmental induction. As shown in Fig. 3D, WT strain began accumulating vexA mRNA at 0 h, with a peak at 24 h post induction, while the DcpcB mutant exhibited a very high level of vexA mRNA at 0 h, with a peak at 24-48 h and 120 h, suggesting that CpcB is needed for proper down-regulation of vexA during sexual development, which might be critical for proper progression of sexual fruiting. Another velvet regulator VosA is a key regulator of sporogenesis, which couples sporogenesis and focal trehalose biogenesis and is required for viable ascospore formation [35,39]. Importantly, as shown in Fig. 3D, whereas WT strain began accumulating vexA mRNA at 48 h post induction of sexual development, the DcpcB mutant did not show any signs of vexA mRNA accumulation. These results indicate that CpcB plays an important role in controlling sexual development and expression of the velvet regulators, and that SfaD (the canonical Gfb) and CpcB (a Gfb-like protein) govern distinct stages of sexual fruiting in A. nidulans [11]. It has been proposed that SfaD (with GpgA) acts on the initiation of sexual fruiting, whereas CpcB functions during meiosis and ascosporogenesis [11,13,24].

The Role of CpcB in Conidial Germination and Cellular Growth

The fact that cpcB mRNA is present abundantly in A. nidulans conidia, and accumulates to high levels during the entire phase of
vegetative proliferation in both species (6 h, Fig. 1A and 1B) led us to examine whether the absence of CpcB would affect conidial germination and hyphal growth. As shown in Fig. 4A, WT A. nidulans conidia showed about 54% germination at 4 h, and 100% germination at 5 h. On the contrary, the ΔcpcB mutant conidia showed 1.7 ± 0.57 μm at 4 h, and 16.4 ± 6.11 μm at 6 h. As the cpcB mutant hyphae do not show differences in the width, these data suggest that CpcB is required for proper cellular growth, and conidial germination in A. nidulans.

Likewise, a severe retardation of conidial germination and cellular growth was observed in the A. fumigates cpcB deletion mutant (Fig. 4B). WT A. fumigates conidia showed about 56% germination at 5 h and 100% germination at 7 h in MM. The average length of hyphae of WT A. fumigates strain was 4.63 ± 2.21 μm at 6 h and 14 ± 1.93 μm at 8 h. On the contrary, the ΔΔfucpB mutant conidia showed 0% germination at 5 h and ~46% germination at 7 h in MM. The average hyphal length of the ΔΔfucpB mutant was 1.25 ± 0.3 μm at 6 h and 8.25 ± 1.71 μm at 8 h. In both species, after 1 day incubation on MMG WT and C’ strains produced a higher number of conidiophores than the cpcB deletion mutants (Fig. 4C and 4D). Collectively, these data indicate that CpcB plays a positive role in spore germination, cellular growth and conidiophore formation in both Aspergillus species.

Role of CpcB in Mycotoxin Production

Previously we showed that SfaD, GpgA and PhnA (a phosducin-like protein) are necessary for the expression of aflR encoding the transcriptional activator for the ST biosynthetic genes and subsequent ST biosynthesis [14]. We checked whether the absence of CpcB also affects ST biosynthesis in A. nidulans. As shown in Fig. 5A, compared to WT and C’ strains, two independent ΔcpcB mutant strains produced reduced amounts of ST and additional unknown compounds with higher Rf values (spots above ST).
Production of ST requires coordinated expression of the pathway-specific transcription factor (AflR) and many biosynthetic enzymes including StcU [49]. We found that DcpcB did not affect mRNA accumulation of aflR and stcU (Fig. 5C; [49]) during asexual developmental induction. These results suggest that reduced production of ST by DcpcB is not caused by the lack of proper gene expression as observed in SfaD and PhnA mutant, but by an indirect effect [14].

A. fumigatus produces the non-ribosomal peptide toxin GT [50], which has immunosuppressive activity and contributes to the virulence of the fungus [51,52]. Previously we showed that SfaD and GpgA are needed for the proper production of GT in A. nidulans [21]. To test whether CpcB is associated with production of GT, we examined GT levels in WT, DcpcB null and DcpcB complemented strains. As shown in Fig. 5B, whereas WT and complemented strains all produced detectable amount of GT, none of the three DcpcB null mutants exhibited the GT production. These results indicated that, like SfaD, CpcB is required for proper production of GT.

CpcB is Needed for Vegetative Growth in the Absence of FlbA or RgsA

FlbA is an RGS protein that rapidly turns off growth signaling mediated by FadA and SfaD[GpgA, and the deletion of flbA results in hyper-active hyphal proliferation followed by autolysis in A. nidulans (see Introduction). We asked whether CpcB is also associated with FadA-mediated growth signaling as a non-canonical GB subunit. This question was addressed by generating the DflbA DcpcB double mutant (QK3) and comparing the resulting phenotypes with the DflbA single mutant (QK1, Table 1). As shown in Fig. 6A, the DflbA DcpcB double mutant exhibits severely impaired colony growth and the lack of autolysis even at 7 days (data not shown). These data indicate that CpcB is necessary for proper vegetative growth even in the absence of the key negative regulator FlbA of growth signaling, and implying that CpcB-mediated proliferation control might be independent of FadA. This is further supported by the fact that the DgpaA DcpcB mutant displayed highly restricted colony growth, produced fewer conidia with enhanced pigmentation (Fig. 6B). Moreover, different from the DflbA DsfaD and the DflbA DgpgA mutants, the DflbA DcpcB double mutant failed to produce abundant conidiophores. This is consistent with our finding that CpcB is required for proper conidiation in Aspergillus, whereas SfaD negatively regulates conidiation [11]. These results indicate CpcB is a critical component of vegetative growth and development, likely independent of FadA and GanB signaling.

Figure 4. The roles of CpcB in conidial germination and hyphal growth. (A—B) Quantitative analyses of conidial germination and hyphal growth post conidial germination of WT (filled bar; TNJ36), DcpcB (blank bar; RJMP1.59-8) and complemented (C) (shaded bar; RJMP1.59-8C) A. nidulans strains on MMG (A); and of WT (AF293), DAfucpcB (AF293.17C) and complemented (C; AF293.17C) A. fumigates strains on MMG (B). (C) Photographs of the three A. nidulans strains grown on solid MMG for 1 day (top panels) and the close-up views (bottom panels). Note the lack of conidiophores in DcpcB strain. (D) Photographs of the three A. fumigates strains of grown on solid MMG for 1 day (top panels), and the close-up views (bottom panels). Note the differences in the size and number of conidiophores.

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In ascomycetous fungi, the biosynthesis of amino acids or charged tRNAs are coordinately controlled by a complex transcriptional network called ‘cross-pathway control’ \[53,54\]. The amino acid starvation signal is perceived by the cross pathway regulatory network and results in increased expression of a transcriptional activator encoded by a c-Jun homologue, GCN-4, cpc-1 \[55\]. In the presence of amino acids, the RACK1 homolog CpcB is required to repress this network. The *A. nidulans* CpcB is shown to function in repressing expression of *argB*, and progression and completion of sexual fruiting.

In this study, we further characterized CpcB’s role in governing growth and development in two *Aspergillus* species. Similar to Gib2, CpcB contains the seven WD-40 repeat motif and is predicted to form a seven-bladed β propeller structure characteristic of β transducins \[22\]. Whereas *C. neoformans* Gib2 is known to interact with two Gγ subunit homologs, Ggp1 and Ggp2, similar to the conventional Gβ subunit Gpb1 \[22\]. *Aspergillus* genomes contain only one probable Gγ GgpA, suggesting that CpcB might interact with GgpA only.

Our central hypothesis for the present study was that CpcB plays a crucial role in governing vegetative growth, spore germination and development in *A. nidulans* and *A. fumigatus*. In *A. nidulans*, while radial growth rates of the *sfaD* and *gpgA* deletion mutants of *A. nidulans* grown on solid medium were similar to those observed for WT strains \[11,13\], those of the ΔcpcB mutant were severely impaired resulting in extremely small colonies (Figs. 2A & 3A), which resembles the mutant lacking the putative GEF RicA \[56\]. In contrast, in *A. fumigatus*, whereas the ΔcpcB deletion mutant exhibited radial growth that is about 80% WT, the ΔAfusfaD and ΔAfugpgA mutants showed extremely impaired hyphal growth on solid medium (Fig. 3A & B; \[21\]). These suggest that, despite the very high level of amino acid identity of CpcB and SfaD between the two species, the primary signaling element for hyphal growth can be different. However, in both species, conidial germination has been hampered by the absence of CpcB or SfaD (Figs. 4A & B;

![Figure 5. CpcB is not required for ST biosynthesis.](image_url)
Figure 6. Phenotype of the ΔflbA ΔcpcB and ΔrgsA ΔcpcB mutants. Photographs of colonies of WT (FGSC4), ΔcpcB (RJMP1.59-8), ΔflbA (QK1), and ΔflbA ΔcpcB (QK3) strains (A); and ΔrgsA (QK2) and ΔrgsA ΔcpcB (QK4) strains (B) grown on solid MMG for 3 days are shown. The two left panels show the point-inoculated strains (top and bottom), and the two right panels show close-up views of single and double mutant colonies shown in the left panels. Photomicrographs were taken by Zeiss Axioplan 2 stereomicroscope. Note the highly restricted growth of the double mutants (A and B), and the lack of conidiation in the ΔflbA and ΔflbA ΔcpcB mutant (A).

Figure 7. A speculative model summarizing the roles of CpcB and SfaD in governing various biological processes in A. nidulans.

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of \( \text{cpcB} \) and negative control of \( \text{Hülle} \) cell formation, as the allele, indicating that \( \text{CpcB} \) plays an essential role in ascosporogenesis in oscillation between ON and OFF status, and regulates cAMP production. Our previous study indicated that \( \text{Gib2} \) likely stabilizes \( \text{Gpa1} \) and facilitates its result in drastically altered upstream signaling, which in turn result in the absence of \( \text{cpcB} \), it is premature to devise the signal transduction cascade(s) associated with \( \text{CpcB} \). Previously, we speculated that \( \text{MAP kinase(s)} \) might be involved in transducing \( \text{SfaD:GpgA-mediated signals for sexual reproduction} \) [13,14]. In accordance with this hypothesis, a recent study revealed that the MAP kinase \( \text{MpkB} \) is required for sexual fruiting. The deletion of \( \text{mpkB} \) resulted in pleiotropic effects including the lack of cleistothecia formation under any induction conditions for sexual development, increased \( \text{Hülle} \) cell production, reduced hyphal growth and aberrant conidiophore morphology [37]. These phenotypes are almost identical to those caused by the absence of \( \text{sfaD} \) or \( \text{cpcB} \). These led us to speculate that \( \text{SfaD} \) and \( \text{CpcB} \) mediated signaling might be in part transduced through \( \text{MpkB} \). The other key downstream components of vegetative growth and developmental regulation are cAMP-dependent protein kinases (PKA), \( \text{PkaA} \) and \( \text{PkaB} \) [3]. \( \text{PkaA} \) is the primary PKA that positively functions in vegetative growth and spore germination, but negatively controls conidiation and ST production [3,14]. Whereas the deletion of \( \text{pkaB} \) alone does not cause apparent phenotypic changes, the absence of both \( \text{pkaB} \) and \( \text{pkaA} \) is lethal, i.e., \( \text{PkaB} \) and \( \text{PkaA} \) are essential for viability of \( \text{A. nidulans} \). Overexpression of \( \text{pkaB} \) enhances hyphal proliferation and rescues the growth defects caused by \( \Delta \text{pkaA} \), indicating that \( \text{PkaA} \) plays a positive role in vegetative growth signaling [58]. Lafon et al. [10] also showed that \( \text{GanB-SfaD::GpgA-mediated glucose sensing and germination signaling} \) is through \( \text{PkaA} \). Collectively, we speculate that \( \text{CpcB} \) and \( \text{SfaD} \) mediated signaling for vegetative growth may involve both \( \text{MpkB} \) and \( \text{PKA} \), whereas their signaling for sexual development might be primarily associated with \( \text{MpkB} \). A speculative model summarizing the potential roles of \( \text{CpcB} \) and \( \text{SfaD} \) in governing various biological processes in \( \text{A. nidulans} \) is presented (Fig. 7).

Supporting Information

Figure S1 Northern blot analyses for levels of \( \text{brlA} \), \( \text{abaA} \), \( \text{wetA} \), \( \text{vosA} \), \( \text{nsdD} \) and \( \text{veA} \) transcripts upon asexual and sexual developmental induction of WT (FGSC4) and \( \Delta \text{cpcB} \) (RJMP1.59-8) strains of \( \text{A. nidulans} \). C indicates conidia, and the numbers indicate time post developmental induction. (TIF)

Table S1 Oligonucleotides used in this study. (DOCX)

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Author Contributions

Conceived and designed the experiments: QK SCK JHY. Performed the experiments: QK LW ZL NJK. Analyzed the data: QK SCK JHY. Contributed reagents/materials/analysis tools: SCK. Wrote the paper: QK LW JHY.
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