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Basal expression of the *Aspergillus fumigatus* transcriptional activator CpcA is sufficient to support pulmonary aspergillosis

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Abstract

Aspergillosis is a disease determined by various factors that influence fungal growth and fitness. A conserved signal transduction cascade linking environmental stress to amino acid homeostasis is the Cross-Pathway Control (CPC) system that acts *via* phosphorylation of the translation initiation factor eIF2 by a sensor kinase to elevate expression of a transcription factor. Ingestion of *Aspergillus fumigatus* conidia by macrophages does not trigger this stress response, suggesting that their phagosomal microenvironment is not deficient in amino acids. The *cpcC* gene encodes the CPC eIF2α kinase, and deletion mutants show increased sensitivity towards amino acid starvation. CpcC is specifically required for the CPC response but has limited influence on the amount of phosphorylated eIF2α. Strains deleted for the *cpcC* locus are not impaired in virulence in a murine model of pulmonary aspergillosis. Accordingly, basal expression of the Cross-Pathway Control transcriptional activator appears sufficient to support aspergillosis in this disease model.

Keywords: Amino acid homeostasis; eIF2α kinase; *cpcC*; Cross-Pathway Control

1. Introduction

Besides bacteria, fungal species represent the second-biggest group of pathogens that are able to infect humans (Woolhouse, 2006), and therefore it is of future interest to elucidate the mechanisms of disease caused by fungal pathogens thoroughly. Pathogenicity often is a multi-factorial trait composed of a variety of factors that contribute to the virulence of a fungal organism (Casadevall, 2006). Characteristics of this so-called virulome determine the outcome of a pathogen-host encounter in strict dependency of the host's immune status, as it has comprehensively been depicted by the damage-response framework (Casadevall and Pirofski, 2003).

Within this concept, fungi of the genus *Aspergillus* represent a distinct category as they can harm an infected individual by eliciting a strong, allergic immune response, or when the major defence lines of innate immunity are impaired. Especially, the latter scenario may result in severe and fatal forms of so-called aspergillosis, which are characterised by invasive and disseminated progressions. The predominant species to cause aspergillosis is the deuteromycete *Aspergillus fumigatus*, but also other aspergilli have been reported in recent studies to cause this disease (Walsh and Groll, 2001; Brakhage, 2005). In general, *Aspergillus* inhabits organic substrates in decaying matters to lead a saprobic lifestyle, and based on the hypothesis that selective environmental pressure forms a

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facultative pathogenic fungus (Casadevall et al., 2003), this ecological niche is likely to have shaped the virulence of *A. fumigatus*. Accordingly, thermophyly, stress resistance, and nutritional versatility are crucial for *Aspergillus* pathogenicity as they support survival and propagation in an alternative ecological niche, the human host.

In eukaryotes, resistance against environmental stress conditions is conferred in part by a well-conserved signal transduction cascade that acts on the level of protein synthesis. In this integrated stress response network, diverse conditions of stress are perceived by various kinases that phosphorylate the α subunit of an initiation factor for translation, eIF2 (Harding et al., 2003; Wek et al., 2006). This in turn lowers cellular rates of translation initiation except for distinct mRNAs that code for regulatory molecules like transcription factors. Consequently, expression of such a terminal effector is increased to generate a cellular counter-reaction to the triggering stress condition. Higher eukaryotes express up to four types of eIF2α kinases, each responding to distinct signals, whereas in the model ascomycete *Saccharomyces cerevisiae* only one such sensor kinase, Gcn2p, has been identified (Wek et al., 1995). The primary signal Gcn2p responds to is amino acid starvation, and the regulatory network constituted by this eIF2α kinase and its effector Gcn4p has been coined as General Control (GC) of amino acid biosynthesis. In filamentous fungi, as *Neurospora crassa* or *Aspergillus* spp., the homologous system is called Cross-Pathway Control (CPC) (Carsiotis and Jones, 1974; Carsiotis et al., 1974; Wanke et al., 1997; Hoffmann et al., 2001). In general, binding of accumulated uncharged tRNA molecules, that mirror amino acid starvation, to the GC/CPC sensor kinase GCN2 results in activation of an intrinsic protein kinase activity to phosphorylate the α subunit of the trimeric eIF2 complex at a highly conserved serine residue (Ser-51). As a result, translation of a transcriptional activator, Gcn4p/CpcA, is elevated and generates a global cellular response. Characterisation of the corresponding transcriptional effector in *A. fumigatus*, the *cpcA* gene product, has led to several conclusions: first, CpcA is a functional orthologue of yeast Gcn4p; second, it is strictly required for the CPC response upon amino acid starvation conditions; third, mutants of *A. fumigatus* deleted for the coding sequence are attenuated in virulence, as monitored in an animal model for pulmonary aspergillosis using neutropenic mice, but show no obvious growth phenotype *in vitro* (Krappmann et al., 2004).

As regulation of CpcA expression in *A. fumigatus* is likely to be affected by an upstream eIF2α kinase and may be accordingly complex, the precise role of this transcriptional activator as a virulence determinant remains to be specified. To address this issue and to answer the question whether a de-repressed CPC system is required for aspergillosis, we aimed at the *A. fumigatus* CPC eIF2α kinase CpcC. Mutant strains lacking the *cpcC* gene product were generated and characterised with respect to amino acid starvation conditions, the CPC response, and virulence.

2. Material and methods

2.1. Strains, media, and growth conditions

Bacterial strains were *Escherichia coli* DH5α [F^-, F80d/*lacZ*M15, Δ(*lacZYA-argF*)U169, *recA1*, *endA1*, *hsdR17* (r_{K-}, m_{K+}), *supE44*, λ^-, *thi1*, *gyrA96*, *relA1*] (Woodcock et al., 1989) for general cloning procedures and KS272 [F^-, Δ*lacX74*, *galE*, *galK*, *thi1*, *rpsL*, Δ*phoA*(PvuII)] carrying the pKOBEG plasmid for recombineering (Chaveroche et al., 2000). *E. coli* strains were propagated in LB or LBLS (1% bacto-tryptone, 0.5% yeast extract, 1% or 0.5% NaCl, pH 7.5) medium. Fungal strains used in this study are listed in Table 1. Growth of *A. fumigatus* strains was carried out at 37 °C on minimal medium prepared and supplemented according to Käfer (1977). Antibiotics' concentrations were 100 μg/ml for ampicillin, 25 μg/ml for chloramphenicol, and 20 μg/ml for phleomycin.

2.2. Transformation procedures

Protocols for *E. coli* were either for calcium/manganese-treated cells (Hanahan et al., 1991) or for electroporation (Dower et al., 1988) with a BIO-RAD GenePulser at 2.5 kV in 0.2 cm cuvettes. *A. fumigatus* was transformed by polyethylene glycol-mediated fusion of protoplasts as described (Punt and van den Hondel, 1992).

2.3. Manipulation of nucleic acids and plasmid constructions

Standard protocols of recombinant DNA technology were carried out (Sambrook et al., 1989). Pfu proofreading polymerase was generally used in polymerase chain reactions (Saiki et al., 1986) and essential cloning steps were verified by sequencing on an ABI PRISM 310 capillary sequencer. Fungal genomic DNA was prepared according to Kolar et al. (1988), and Southern analyses were carried out essentially as described (Southern, 1975). Total RNA samples were isolated employing the TRIzol reagent of INVITROGEN followed by Northern hybridisation according to the protocols cited by Brown and Mackey (1997). Random primed labelling was performed with the STRATAGENE PRIME-IT® II kit in the presence of [α-^{32}P]-dATP (Feinberg and Vogelstein, 1983). Autoradiographies were produced by exposing the washed membranes to KODAK X-OMAT films.

Plasmids used and constructed during the course of this study are listed and briefly described in Table 1, together with essential oligonucleotides employed to construct them. pME3286 served as reference for the *cpcC* sequence and contains a genomic DraI/NaeI fragment cloned in the EcoRV site of the general cloning plasmid pGEM5(+). To construct a deletion cassette for the *cpcC* locus, a suitable template was constructed as follows: a 3.1 kb FspI/SpeI fragment comprising the *cpcC* 5′ region was inserted into pGEM5(+) via EcoRV/SpeI, followed by insertion of a 3′ PstI fragment (5.1 kb) into the Mph1103I site. The resulting plasmid pME3287 was digested with NdeI and

Table 1
Fungal strains, plasmids, and oligonucleotides used in this study

Name	Description or sequence	Reference
Strains		
D141	*Aspergillus fumigatus wild-type* strain (syn. NRRL 6585), clinical isolate	Staib et al. (1980)
AfS01	Strain D141 carrying deletion of *cpcA* coding sequence: Phleo^r, MSX^s	Krappmann et al. (2004)
AfS26	Strain D141 carrying deletion of *cpcC* coding sequence: Phleo^r, MSX^s	This study
AfS27	AfS26 with excised marker module: Phleo^s, MSX^s	This study
AfS55	Reconstituted AfS01 expressing *gfp*-tagged *cpcA* allele: Phleo^s, MSX^r	This study
Plasmids		
pBluescript II KS	General cloning plasmid [*bla*, multiple cloning site]	STRATAGENE
pGEM5(+)	General cloning plasmid [*bla*, multiple cloning site]	PROMEGA
pPTRII	Autonomously replicating Aspergillus plasmid [*ptrA*, *AMA1*, *bla*]	TAKARA
pKOBEG	pSC101 derivative expressing λ phage *red*γβα operon from pBAD promoter	Chaveroche et al. (2000)
pMCB17	GFP gene (*pyr-4::^{p}alcA::gfp2-5*) in pUC19	Fernandez-Abalos et al. (1998)
pME2563	*cpcA* allele carrying silently mutated *Sal*I site in coding sequence	Krappmann et al. (2004)
pME2891	*loxP-phleo^r/tk* blaster [*loxP-^{p}gpdA::ble/HSV1 tk::trpC^t-loxP*]	Krappmann et al. (2005)
pME2892	Cre expression module in pPTRII [*A. nid. niaD::cre*, *ptrA*, *AMA1*]	Krappmann et al. (2005)
pME3286	Genomic *cpcC* locus as 6.1 kb DraI/NaeI fragment in pGEM5(+) EcoRV	This study
pME3287	Template for construction of *cpcC* deletion cassette by recombinatorial cloning	This study
pME3288	*cpcC::loxP-phleo^r/tk-loxP* replacement cassette for complete deletion	This study
pME3289	Construct for *cpcA*Δ reconstitution by *gfp::cpcA* allele	This study
Oligonucleotides		
Sv117	5′-AGA CCT ACT GAA TCT GGG TTC AGA CTT TCT ACA CCG CCC ACC GCC CCG CTC AGC TGA AGC TTC GTA CGC-3′	
Sv118	5′-ACT TCA ATA CAA ATA GAA CAT AAC ATG GTA CTT CGT CAC TCG TTG ATC CTG CAT AGG CCA CTA GTG GAT CTG-3′	
Sv319	5′-ATC TTT GTC ACT CGC GTC TCA CG-3′	
Sv320	5′-TCT GCA CGC TGA TTG GCA GCA GC-3′	
Sv321	5′-TGA TTT ATG AAC TCG CCG TGT CG-3′	
Sv322	5′-ACG CTC CCC ACG ATG TCC AGG-3′	
Sv323	5′-ATT GGT TTC AAG ATA TCT AGG-3′	
Sv324	5′-ATC AAA GTC TTG GTT GTA TCG-3′	
Sv325	5′-ACA CAC CTT TGA TTG CTC GCA C-3′	
Sv326	5′-TCT GGT TTC AGA TCG CGG TGG-3′	
Sv327	5′-TGA TGC AAG CCT ATC TCA AGG-3′	
Sv328	5′-ACT GCC TTT TCC GAG TAG TGG-3′	
Sv329	5′-TTG GAA CTA CTT ACT ATG TCG-3′	
Sv330	5′-TTC TGG GCT CGC TGC CAT GG-3′	
Sv331	5′-TCA CTA GAG AAA ACA TTC GC-3′	
Sv332	5′-ATG TGT TTG AGA GCA GCT CGC-3′	
Sv333	5′-AAT GTG TTT TTG ACA CTA AGC-3′	
Sv334	5′-TTC TGT TCG TCT TCT TGC TCC-3′	
Sv335	5′-ACC TAC GGC AGT CAA GAT TGC-3′	
Sv336	5′-TAC TTC GTC ACT CGT TGA TCC-3′	
CS41	5′-TAT AGT CGA CCA GTA AAG GAG AAG AAC TT-3′	
CS42	5′-TAT ACT CGA GTA TTT GTA TAG TTC ATC CAT-3′	

NotI, and the resulting backbone was co-transformed with a PCR amplicon from pME2891 with primers Sv117/118 into the *E. coli* recipient KS272 replicating pKOBEG (Chaveroche et al., 2000). The resulting construct from this recombinering step is pME3288, which served as replacement cassette for the *cpcC* coding sequence. For N-terminal tagging of the *cpcA* coding region, the *gfp2-5* sequence was amplified with primer pair CS41/CS42 to become inserted after SalI/XhoI digestion into the SalI site of pME2563, yielding pME3289. From this construct, a 9 kb fragment was released *via* BssHII to reconstitute the *cpcA* locus in deletion strain AfS01.

For determination of the actual *cpcC* coding sequence, reverse transcription from an oligo(dT) primer (MBI FERMENTAS) was carried out on total RNA that had been isolated from vegetative D141 cultures, pairs of oligonucleotides

(Sv319–Sv336) were used to amplify suitable stretches spanning predicted intronic regions from this template, and the resulting cDNA amplicons were directly sequenced.

2.4. Biochemical methods and western blots

Crude extracts were prepared by grinding washed and shock-frozen mycelia to a fine powder and extracting soluble proteins with buffer (100 mM Tris–HCl, 200 mM NaCl, 20% glycerol, 5 mM EDTA, pH 8) at 4 °C in the presence of the protease inhibitor phenylmethylsulfonylflouride. Protein contents were determined by the procedure of Bradford (1976). Enzymatic activities of ornithine carbamoyltransferase (OTCase, E.C. 2.1.33) activities were determined according to Tian et al. (1994). Western analysis of eIF2α phosphorylation was carried out essentially as described earlier (Grundmann et al., 2001) with a polyclonal anti-eIF2α antibody (provided by Alan Hinnebusch; Romano et al., 1998) and a phosphorylation-specific polyclonal anti-eIF2α ~ P antibody (BIOSOURCE International, Camarillo, CA, USA). Cross-reactions were visualised using the ECL technology (AMERSHAM PHARMACIA Biotech).

2.5. Virulence tests in a murine model of pulmonary aspergillosis

Outbred male mice (strain CD1, 20–28 g, Charles Rivers Breeders) were used for animal experiments. Immunosuppression was executed with hydrocortisone acetate (112 mg/kg subcutaneous) and cyclophosphamide (150 mg/kg intraperitoneal) following the protocol of Smith et al. (1994), bacterial infections were prevented by adding tetracycline (1 g/l) and ciproxin (64 mg/l) to the drinking water. Inocula of up to 2×10^5 conidiospores in 40 μl of saline were prepared by harvesting spores from 5-day-old slants of solid medium followed by filtration through miracloth and washing with saline (Tang et al., 1993; Aufauvre-Brown et al., 1998). Mice were anaesthetized by inhalation of halothane and infected by intranasal instillation. The weights of infected and control animals were monitored for up to 10 days twice daily and mice developing severe pulmonary illness, characterized by respiratory distress, hunched posture and poor mobility, or 20% weight loss were culled. Lungs of these animals were homogenized in saline and aliquots were spread on standard medium to check for fungal growth.

2.6. Cell preparations

Cells for confrontation assays were essentially prepared as described by Gunzer and co-workers (Behnsen et al., 2007): murine alveolar macrophages were obtained by washing the trachea and lungs of BALB/c mice with PBS through a 22G plastic catheter to obtain bronchoalveolar lavage fluid. After erythrocyte lysis, the cells were resuspended in complete medium supplemented with glutamine, penicillin, and streptomycin and kept on ice until further use; J774 cells were cultured in BioWhittaker's X-Vivo 15 medium.

2.7. Fluorescence and light microscopy

Conidia and mycelia were examined with a Zeiss Axiovert microscope by either differential interference contrast microscopy (DIC) or fluorescence microscopy using a GFP filter set (AHF Analysentechnik AG, Tübingen, Germany). Photographs were taken using a Xillix Microimager digital camera and the Improvision Openlab software (IMPROVISION, Coventry, UK).

3. Results

3.1. Phagocytosis by macrophages does not trigger CpcA expression in Aspergillus fumigatus

To gain information on any requirement of balanced amino acid homeostasis in pathogenicity of *A. fumigatus*, we were interested whether phagocytosis of conidia by immune effector cells would elicit a Cross-Pathway Control response. For that purpose, a suitable reporter strain, AfS55, was constructed that expresses a functional *gfp::cpcA* allele from the native gene locus. Induction of the CPC signal transduction cascade ultimately results in increased levels of this transcriptional activator, and in *in vitro* control experiments a clear nuclear fluorescence was evident in this strain when starved for amino acids (Fig. 1a). When confronted with cells of the J774 macrophage cell line, freshly harvested conidia from strain AfS55 were readily ingested and lysed in the phagolysosomal compartment. However, no lucid CpcA expression could be detected within these spores during the intracellular killing (Fig. 1b). However, when phagocytosis was monitored in the presence of amino acid starvation, as induced by the histidine analogue 3-aminotriazole (3AT), nuclear fluorescence was evident, thus demonstrating validity of the assay. Moreover, when murine alveolar macrophages were used in the confrontation experiment, no fluorescence and therefore no expression of the CPC transcriptional activator could be monitored.

To support this observation, immunocompetent mice were infected with conidia from AfS55, and fluids from bronchoalveolar lavages were rescued after four hours containing a mixture of alveolar macrophages and ingested as well as free conidia. These samples were subjected to microscopy to show no fluorescence in the samples from infection experiments with untreated AfS55 conidia (Fig. 1c). Also, no fluorescence could be detected throughout when spores from the *wild-type* isolate D141 were used, but samples from control infections with conidia from a strain expressing the GFP tag constitutively displayed a clear fluorescent signal (not shown). Accordingly, this demonstrates that the environment encountered by *A. fumigatus* during pulmonary infection does not trigger the Cross-Pathway Control response.

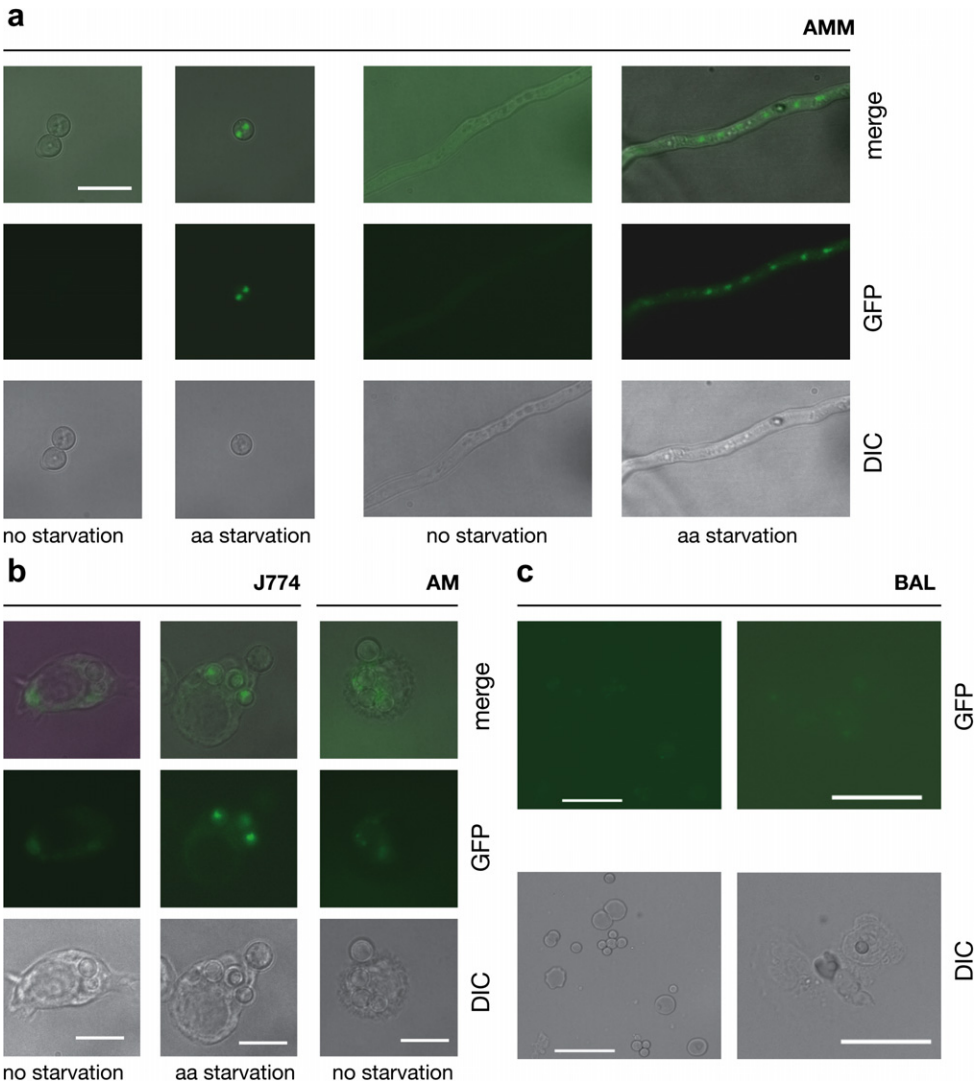

Fig. 1. The Cross-Pathway Control response in *A. fumigatus* conidia is not de-repressed during confrontation with macrophages or infection of immunocompetent mice. Shown are representative images of the *A. fumigatus* Cross-Pathway Control reporter strain AfS55 [*gfp::cpcA*] propagated in *Aspergillus* minimal medium (AMM, a) or in cell culture medium and challenged with phagocytic cells, such as the macrophage cell line J774 or alveolar macrophages (AM, b), or when rescued after bronchoalveolar lavage (BAL, c). Starvation for the amino acid histidine to de-repress the CPC system was induced by adding the false feedback inhibitor 3-aminotriazole. Only when starved for histidine, clear nuclear fluorescence of AfS55 is evident, whereas phagocytosis *ex vivo* or *in vivo* does not elicit expression of the GFP::CpcA reporter construct. DIC: difference interference contrast; scale bars represent 10 μm.

3.2. The Aspergillus fumigatus genome encodes a Gcn2p-like eIF2α kinase

Eukaryotic eIF2α kinases are characterised by their modular structure and contain conserved sensory, regulatory, and structural domains that flank a serine/threonine protein kinase function. In order to identify a Cross-Pathway Control sensor kinase of *A. fumigatus*, the genome sequence of the isolate Af293 was screened with orthologous sequences from other fungi, with the *S. cerevisiae* Gcn2p sequence serving as a structural prototype. BLAST searches revealed an annotated gene locus (Afu5g06750) with a high degree of conservation and significant similarity with respect to known fungal GC/CPC sensor kinases (Fig. 2). Besides the characteristic catalytic domain found in such kinases, several subdomains are present in the deduced gene product of this locus: a histidyl-tRNA synthetase-related region, which is located C-terminal to the eIF2α kinase domain, that mediates tRNA binding, a region resembling a degenerated kinase domain, and a C-terminal region required for ribosome association and dimerisation (see below). The identified *A. fumigatus* gene encoded by this locus was designated *cpcC*, which is in agreement with the nomenclature of CPC genes identified in the filamentous ascomycetes *A. nidulans* and *N. crassa* (Wanke et al., 1997; Sattlegger et al., 1998). To confirm its genomic architecture as predicted by the automatic annotation procedure, the complete genomic *cpcC* locus was isolated from a suitable genomic sub-library and cloned in plasmid pME3286, and in parallel the sequence

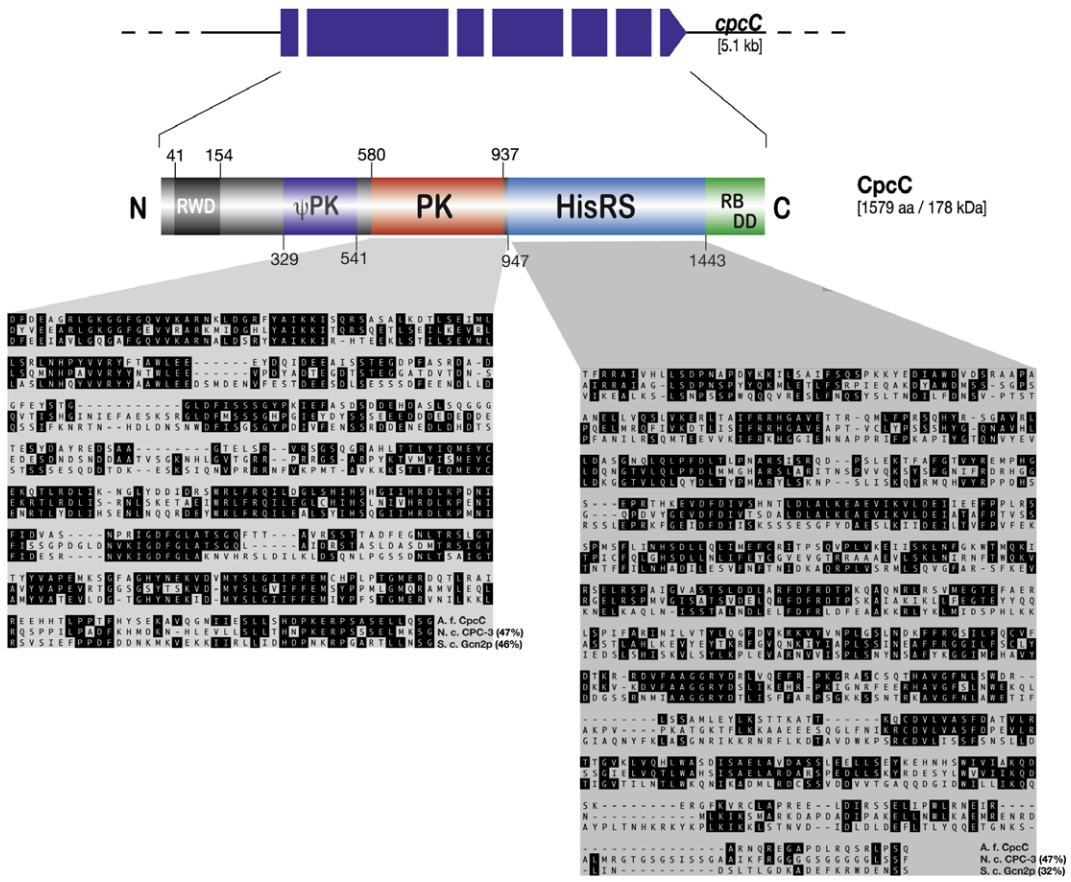

Fig. 2. Architecture of the *cpcC* gene from *A. fumigatus*. Schematic illustration of the *A. fumigatus cpcC* gene locus with seven exons (blue boxes) interrupted by six introns; the coding region spans 5.1 kb on the chromosome. The deduced gene product is schematically shown underneath: CpcC is a protein of 1579 amino acids and contains several well-conserved elements, such as an RWD domain, a pseudo-kinase domain (ψPK), the actual eIF2α kinase domain (PK) and the sensor domain resembling a histidyl-tRNA synthetase (HisRS) adjacent to it, and the C-terminal part required for dimerisaton (DD) as well as ribosome association (RB). Positions of respective domains are given as they were deduced from multiple alignments. The degree of conservation of the eIF2α protein kinase domain (left) and the HisRS sensor domain (right) that binds uncharged tRNA molecules are shown by multiple primary sequence alignments including fungal CpcC counterparts from *Neurospora crassa* (N. c. CPC-3) and *Saccharomyces cerevisiae* (S. c. Gcn2p) with conserved residues shaded in black, values indicate identity percentages in pairwise alignments among the sub-domains.

of the coding region was determined from its transcript by RT-PCR. The CpcC-encoding sequence is composed of seven exons interrupted by six intronic stretches between 47 and 63 nucleotides (nt) in length. The complete coding sequence spans 4779 basepairs (bp) that correspond to 1593 codons. Accordingly, the deduced gene product has a calculated molecular weight of almost 180 kDa. High similarities between domains of CpcC and that of other fungal GCN2 kinases are evident, making the classification of functional elements in the deduced primary sequence possible: Residues 41 to 154 constitute an RWD domain, which is likely to bind the GCN1/GCN20 complex. The region from position 329 to 541 resembles a kinase domain; however, due to the lack of specific invariant residues required for catalytic activity, this domain is likely to be that of a degenerate, inactive kinase analogous to the *S. cerevisiae* and *N. crassa* GCN2 ψPK domains. Deduced from the sequence alignments and *rpsblast* searches, the catalytic protein kinase domain of CpcC resides between amino acids 580 and 937. As it is typical for eIF2α kinases, two pairs of subdomains (IV–V and IX–X) in the kinase domain are separated by inserts that are variable in length and sequence. Adjacent to the eIF2α kinase domain, the conserved HisRS-like domain (pos. 947 to 1443) can be found that resembles a histidyl-tRNA synthetase and binds together with the C-terminal portion the actual effector molecules, uncharged tRNAs. Moreover, the far C-terminus is required for dimerisation and proper localisation to the ribosome and therefore well conserved among the fungal GCN2-like kinases.

3.3. Deletion of *cpcC* in *A. fumigatus* impairs the Cross-Pathway Control response

To gain information on the cellular function of the *cpcC*-encoded gene product, a procedure to create a deletion mutant was followed. Thus, a suitable deletion cassette for gene replacement by homologous recombination was generated and transformed into the clinical isolate D141, which serves as *wild-type* reference strain (Fig. 3a). Several descendants were sub-cultured from the pool of primary transformants that could be isolated on corresponding

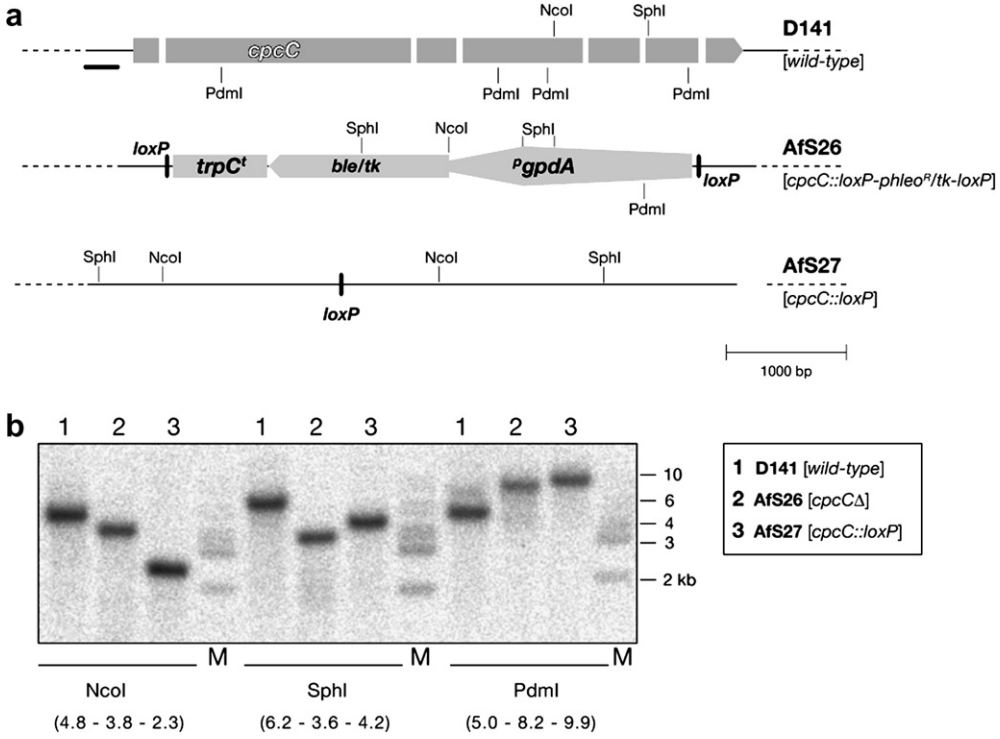

Fig. 3. Generation of *A. fumigatus cpcC*Δ strains. Deletion strains of the *A. fumigatus* wild-type isolate D141 were generated by gene replacement with a recyclable resistance cassette. (a) Outline of the wild-type *cpcC* gene locus in strain D141 and after replacementas present in strain AfS26; additionally, the *cpcC::loxP* locus of descendant AfS27 resulting from marker excision by transient Cre expression is shown. The black bar indicates the region covered by the probe used in Southern analyses. (b) Autoradiography from Southern blot analyses of strains *A. fumigatus* D141, AfS26, and AfS27. The indicated restriction enzymes were used to digest genomic DNA samples and calculated fragment sizes are given underneath; fragment positions from marker (M) lanes are shown on the right hand side.

selective media, and comprehensive Southern analyses confirmed the desired *cpcC*Δ genotype for several of them, from which one representative (AfS26) was chosen for further processing. The strain was transformed with an autonomously replicating plasmid (pME2892) to transiently express the Cre recombinase leading to excision of the resistance marker module (Krappmann et al., 2005). The resulting strain AfS27 was also confirmed for its *cpcC::loxP* genotype *via* Southern blot hybridisation to become included in the following analyses (Fig. 3b).

In a first test the growth behaviour of the *cpcC*Δ deletion mutant was evaluated in the presence of amino acid starvation conditions. For this purpose, strains were inoculated on minimal medium containing the drug methionine sulfoximine (MSX), a glutamine synthetase inhibitor. As reference strains the *wild-type* progenitor D141 as well as a strain lacking the CPC transcriptional activator CpcA (AfS01) were also inoculated (Fig. 4a). Clear differences in hyphal extension and sporulation were evident when amino acid homeostasis was perturbed: whereas the *wild-type* strain displayed proper growth, no growth could be monitored for the *cpcA*Δ deletion strain. Interestingly, the strain ablated for the eIF2α kinase CpcC exhibited retarded but detectable growth on the amino acid starvation medium. Given the fact that D141 and AfS01 express different levels of the transcription factor CpcA in response to CPC derepression—zero in AfS01 [*cpcA*Δ] and high level expression in D141 [*wild-type*]—this observation is in good agreement with the proposed model of CpcA expression (see Section 4).

To evaluate the CPC response of the *cpcC*Δ strain in more detail, steady-state levels of reporter transcripts were determined in Northern blot hybridisations (Fig. 4b). For that purpose, pre-grown mycelia were split and transferred into fresh minimal medium with or without a false feed-back inhibitor of histidine biosynthesis (3-amino-triazole, 3AT) to starve strains for this amino acid. Levels of the chorismate mutase-encoding *aroC* transcript served as internal standard of constitutive expression, as this is an amino acid biosynthetic gene described not to be subject of CPC regulation (Krappmann et al., 1999). In contrast, *argB* transcript levels increased significantly upon the onset of histidine starvation in the *wild-type* isolate, and this representative read-out was absent in the *cpcC*Δ deletion mutant strain. In further hybridisations, transcripts of the genes *cpcA* and *cpcC* were probed to gain additional information on the transcriptional CPC response of the mutant strain. Upon amino acid starvation, transcription levels of the key effector CpcA are strongly increased, which is probably based to a certain extent on positive feed-back regulation; in line with this assumption is the observation that in the mutant strain lacking the CPC sensor kinase this up-regulation of *cpcA* transcription was reproducibly less pronounced although still existent. This is reminiscent to the

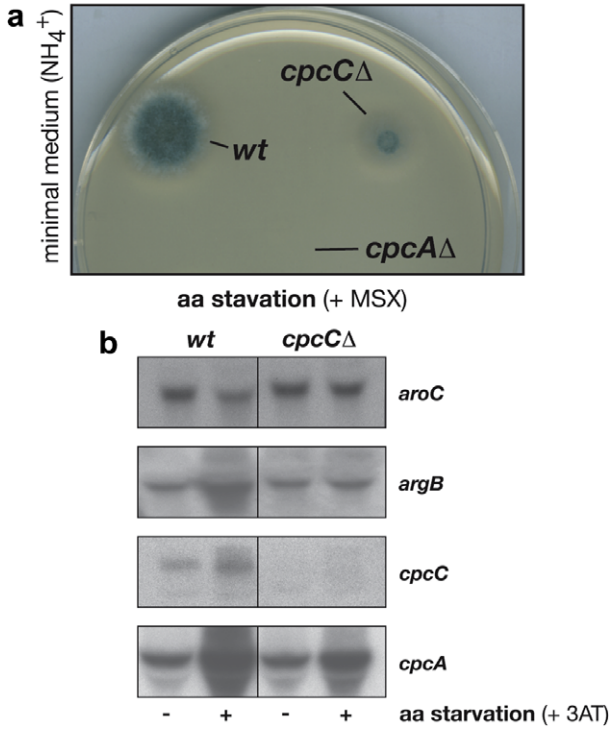

Fig. 4. Phenotypes of a *cpcC*Δ mutant strain under amino acid starvation conditions. Strain AfS26 carrying the resistance marker was used for phenotypic characterisation, which behaved identical to strain AfS27 [*cpcC::loxP*]. (a) Growth behaviour on minimal medium in the presence of glutamine starvation induced by MSX. For comparison, the clinical isolate D141 [*wt*] as well as the deletion strain AfS01 [*cpcA*Δ] ablated for the CPC transcriptional activator is shown. (b) Assessment of the CPC status by Northern analyses. Steady-state transcript levels of the constitutive internal control *aroC* and the CPC target gene *argB* under sated or histidine starvation conditions (+3AT) clearly demonstrate the CPC^- phenotype of the *cpcC*Δ strain. Moreover, hybridisation signals from corresponding probes indicate an increase in *cpcC* transcription under amino acid starvation conditions, and that transcriptional induction of *cpcA* expression depends partially on the presence of the *cpcC*-encoded eIF2α kinase.

situation in the ascomycete *N. crassa* where *cpc-1* transcript levels could be induced by amino acid starvation in a mutant deleted for the orthologous sensor kinase gene *cpc-3* (Sattlegger et al., 1998). Moreover, a slight increase in the transcript levels upon 3AT exposure could also be detected for the *cpcC* gene, indicating that this regulatory gene is part of the *A. fumigatus* CPC transcriptome.

The observed growth phenotype of a *cpcC null* mutant with respect to amino acid starvation together with the data from Northern analyses clearly corroborate that we had identified the CPC sensor kinase and that it is the sole eIF2α kinase required for the Cross-Pathway Control response *in vitro*.

3.4. A cpcC null mutant of A. fumigatus is still able to phosphorylate eIF2α

As the *cpcC* gene is assumed to encode a kinase that acts on the translation initiation factor eIF2, the degree of phosphorylation was followed upon derepression of the CPC system. For this purpose, the cross-reaction of specific antibodies raised against the α-subunit of eIF2 was monitored in Western experiments, and a biochemical assay was employed to validate the CPC status. As expected, phosphorylation of eIF2α increased from a basal level when an *A. fumigatus wild-type* strain was shifted to amino acid starvation conditions using various inhibitors of fungal amino acid biosynthesis (Fig. 5a). In accordance with this result, enzymatic activities of the *argB*-encoded OTCase were elevated (Fig. 5b). To our surprise, the elevated eIF2α-P signal was also clearly and reproducibly detectable from crude extracts that had been prepared from the *cpcC*Δ deletion mutant strain cultivated under identical conditions. OTCase activities determined from these crude extracts, however, revealed no increase and hence confirmed the cpc^- phenotype of the *cpcC*Δ mutant background. Given the high specificity of the phospho-eIF2α antibody, these data suggest that CpcC is not the only eIF2α kinase encoded in the *A. fumigatus* genome, however its action is specific and strictly required for a proper Cross-Pathway Control response of this fungus to counteract amino acid deprivation.

3.5. Virulence of A. fumigatus is not affected in a cpcCΔ background

In a previous study, we had demonstrated that the transcriptional activator of the *A. fumigatus* CPC system is

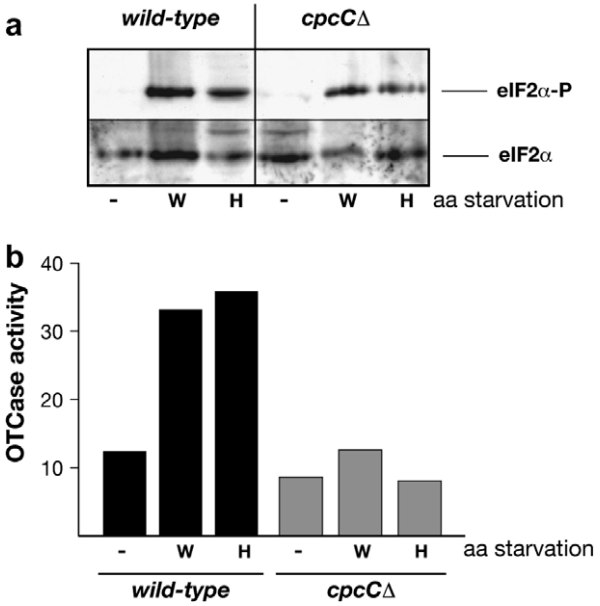

Fig. 5. eIF2α phosphorylation is not abolished in a *cpcC*Δ background. (a) Western experiments using crude extracts from strains D141 [*wild-type*] and AfS26 [*cpcC*Δ] starved for tryptophan (W) by 5-methyltryptophan (5MT) or histidine (H) by 3AT are shown, in which polyclonal antibodies raised against eIF2α were used as internal standard and a specific one to monitor the amount of eIF2α phosphorylated on Ser51. (b) Parallel determination of *argB*-encoded OTCase activities support the cpc^- phenotype of the *cpcC*Δ strain to indicate that CpcC-specific phosphorylation of eIF2α is required for a proper Cross-Pathway Control response.

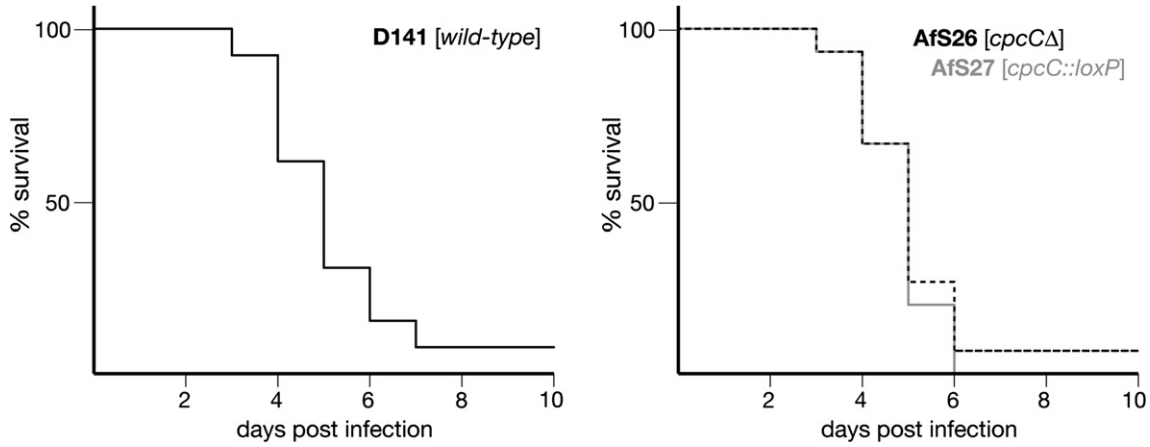

Fig. 6. Virulence of *A. fumigatus cpcC*Δ strains is unaltered in a murine model of pulmonary aspergillosis. Survival plots for groups of leukopenic CD1 mice intranasally infected with $2 \cdot 10^5$ conidia of *A. fumigatus* strains D141 [*wild-type*] (left panel) or *cpcC*Δ deletion mutants (right panel) are shown. Animals received an intraperitoneal dose of 150 mg/kg cyclophosphamide on days −3, −1, 2, and every third day plus a subcutaneous dose of 112.5 mg/kg hydrocortisone acetate on day −1. No virulence differences for this set of strains is could be deduced in this animal model for pulmonary aspergillosis.

required for full virulence in a murine model of pulmonary aspergillosis (Krappmann et al., 2004): mutant strains deleted for the encoding *cpcA* gene appeared attenuated in infection studies but did not show any obvious phenotype *in vitro* except sensitivity towards amino acid analogues. To elucidate whether a derepressed CPC system is necessary for full virulence, both *cpcC*Δ strains AfS26 [*cpcC::loxP-phleo*^{R}/*tk*] and AfS27 [*cpcC::loxP*] were used to infect cohorts of leukopenic mice that had been immunocompromised following a standard protocol using hydrocortisone and cyclophosphamide. Health conditions of the animals were monitored over a time period of up to ten days to assess weight loss or severe signs of pulmonary distress. As reference, again the clinical isolate and progenitor strain D141 was used, which resulted in culling of 12 mice of a 13-animal cohort in the experimental time frame (Fig. 6). Both mutant strains, however, also caused the onset of pulmonary aspergillosis in all infected animals to result in almost exceptionless killing of the experimental groups. Median survival times were in the same range for all three strains tested in this experimental series, so no differences with respect to virulence were evident between the *wild-type* and the *cpcC*Δ mutant background.

4. Discussion

Invasive diseases caused by aspergilli are characterised by the impact of numerous factors that influence the outcome of the fungus/host interaction. It is generally assumed that the natural habitat selects for traits that contribute to the pathogenicity of an opportunistic pathogen. Accordingly, common cellular and physiological attributes represent virulence-determining factors and add to the fungal virulome. Among the most important fungal determinants, nutritional versatility as well as stress resistance have to be considered, as both qualities influence the *in vivo* growth rate, which is directly correlated to virulence (Rhodes, 2006).

The environment encountered by *A. fumigatus* upon infection represents a specific ecological niche that is possibly stressful, especially with respect to nutritional supply, so the pathogenic potential of this particular *Aspergillus* species implies that *A. fumigatus* is well equipped and adapted to utilize the surrounding tissue. To gain insight into the mechanism of aspergillosis, comprehensive knowledge of factors that support infection and *in vivo* growth is required, and therefore metabolic routes that support fungal survival in this possibly hostile environment are of interest. Besides components of primary routes, regulatory cascades that act on clusters of metabolic pathways are informative targets in phenotypic mutant analyses, and one prominent signal transduction pathway that relates environmental stress to fungal physiology is represented by eIF2α kinase signalling. Previous studies have demonstrated that the terminal effector of this cascade, the CpcA transcriptional activator, is required for full virulence of *A. fumigatus* in a murine model of pulmonary aspergillosis (Krappmann et al., 2004). Increased expression of CpcA, however, does not occur upon ingestion by macrophages, which act as primary defence line when spores of this fungal pathogen are inhaled down to the alveoli (Ibrahim-Granet et al., 2003). Accordingly, phagocytosis by macrophages appears not to induce the Cross-Pathway Control system in *A. fumigatus* conidia. This implies that the microenvironment of the macrophage phagosome contains sufficient amounts of amino acids and represents a balanced environment with respect to amino acid homeostasis. This observation is in line with previous studies monitoring the immediate transcriptional re-programming of *Candida albicans* cells after phagocytosis by immune effector cells: ingestion by neutrophils but not macrophages results in an amino acid starvation response by inducing biosynthetic genes of the arginine pathway in a Gcn4p-dependent manner (Rubin-Bejerano et al., 2003). The specific transcriptional response of *C. albicans* after ingestion by macrophages is characterised by induction of alternative

carbon metabolism, enhanced nutrient acquisition, and repression of the translational machinery, but not de-repression of the General Control system (Lorenz et al., 2004). This shift of *C. albicans* confronted with macrophages to a starvation mode that is distinct from the conventional GC/CPC response was recently substantiated by proteome studies (Fernandez-Arenas et al., 2007). In view of that and our macrophage ingestion data with an *A. fumigatus* CpcA reporter strain, an operative Cross-Pathway Control appears obsolete for a fungal pathogen in the course of phagocytosis by macrophages.

In this study, we were able to reveal that the upstream signalling sensor, the eIF2α kinase CpcC, appears to be redundant for pathogenicity of *A. fumigatus*, as indicated by unaffected virulence capacities of corresponding deletion mutant strains in an infection model using leukopenic mice. This kind of model for pulmonary aspergillosis was chosen for several reasons: First, the preceding studies on the *cpcA*Δ mutant had been carried out in the same model, making virulence characteristics of *A. fumigatus cpcA*Δ and *cpcC*Δ mutants comparable. Second, we were interested in virulence based on growth characteristics and the ability to exploit the infected tissue as substrate. In contrast to this, infection models based on hydrocortisone treatment solely do not result in depletion of neutrophils, and there, more subtle effects resulting from the interaction of *A. fumigatus* strains with the host's innate immune system may be gained, which is out of the scope of this particular study. Given the complex mechanism of CpcA expression, the negative result on full virulence of *cpcC*Δ deletants indicates that basal but not elevated levels of this transcription factor are sufficient but also necessary to support virulence of *A. fumigatus*. Transcription from the *cpcA* locus results in mRNA molecules from which CpcA is translated at low levels due to the leakiness of translational barriers in the 5′ leader region. The onset of starvation, which is sensed by the CpcC kinase, relieves the translational block mediated by these upstream open reading frames (uORFs) to result in high levels of CpcA. Accordingly, when the function of the sensor kinase is impaired, no de-repression of the CPC system can occur; however, basal levels of CpcA are steadily expressed due to enduring transcription of the encoding gene. The existence of such basal but un-inducible levels of the transcriptional activator are clearly mirrored by the attenuated, intermediate MSX^S growth phenotype of a *cpcC*Δ mutant. The assumption that the CPC system is not de-repressed during aspergillosis is substantiated by additional studies: first, monitoring *in vivo* levels of *cpcA* transcripts by competitive RT-PCR revealed constant levels of gene expression (Zhang et al., 2005), and second, preliminary *in vivo* transcriptome profiles are clearly distinct from the data set that is generated in response to amino acid starvation (our unpublished results). Conclusively, no impact of the CPC signal transduction pathway on *A. fumigatus* pathogenesis can be deduced, arguing for the absence of nutritional stress conditions with respect to amino acid homeostasis in the murine lung. However, our data do not exclude the presence of a redundant signalling pathway that might function through an alternative sensor kinase. As indicated in our Western experiments monitoring the eIF2α phosphorylation status, additional eIF2α kinase activities are present in *A. fumigatus*. However, action of CpcC is specific for and strictly required for a proper Cross-Pathway Control response of this fungus to counteract amino acid deprivation. Inspection of the *A. fumigatus* genome sequence indeed confirms the existence of a second eIF2α kinase, the *ifkB* (for *i*nitiation *f*actor *k*inase B) gene product. Our preliminary characterisation of this gene and its gene product implies that this kinase is functionally not redundant to CpcC but is, however, responsible for the residual eIF2α phosphorylation in a *cpcC*Δ background: an *ifkB*Δ mutant strain is not impaired in its CPC response and an *ifkB*Δ; *cpcC*Δ double deletion mutant does not display phosphorylation of eIF2α in Western experiments (data not shown). Moreover, no clear cellular role could be assigned to the IfkB kinase through our preliminary phenotypic studies, so it is unclear whether this eIF2α kinase affects virulence of *A. fumigatus* in our murine model of pulmonary aspergillosis.

Assuming that uninduced CpcA levels contribute to virulence of *A. fumigatus* raises the question, which sub-set of genes is targeted and driven in their expression by low CpcA quantities. The existence of such genes could be demonstrated in *S. cerevisiae* (Paravicini et al., 1989) and it is likely that in *A. fumigatus* several genes of that kind exist. Comprehensive profiling data on the CpcA-dependent but CPC-uninduced transcriptome and proteome will assist in defining such a basal targetome of this conserved transcription factor to gain further knowledge on the influence of the Cross-pathway Control system in pathogenicity of *A. fumigatus*.

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