Cross-Pathway Control of the Pathogenic Fungus *Aspergillus fumigatus*: a Manifold Stress Response System

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Summary

In this study the Cross-Pathway Control (CPC) of the opportunistic pathogenic fungus *Aspergillus fumigatus* was analysed in view of putative stress response genes, which enables the fungus to adapt within the host. Previous works displayed that the transcriptional regulator CpcA of the CPC is required for full virulence of *A. fumigatus*.

In the first part of this work an *A. fumigatus wild-type* and its *ΔcpcA* derivative were compared under conditions of amino acid starvation *via* transcriptome profiling. 377 genes were detected that are induced under starvation, and 146 displayed a reduced transcriptional level in comparison to the *ΔcpcA* deletion strain. Classification pointed out that most of the genes are involved in primary and secondary metabolism. In addition, expression of numerous genes encoding transporters and permeases was increased as well as that of some genes coding for transcriptional regulators. Further, twelve regulated genes involved in any kind of stress response like detoxification of oxidative molecules and drug resistance were found. Within these genes, two encoding so-called CpcA-dependent AAA-ATPases (*cdaA* and *cdaB*) could be identified. Deletion of these genes led to an increased sensitivity towards Calcofluor White, which is a cell wall stress-inducing drug. These results clarify the flexibility of the fungus to react on different kind of stress signals *via* activation of the transcriptional regulator CpcA.

The second chapter describes the CPC sensor kinase CpcC and its influence on pathogenicity of *A. fumigatus*. A *ΔcpcC* deletion mutant is unable to induce the expression of CpcA under amino acid starvation conditions. Moreover, the deletion strain displayed no decreased pathogenicity in a murine model of pulmonary aspergillosis. Therefore, it is suggested that the basal level of CpcA seems to be sufficient for full virulence. This finding is supported by the fact that during the early phase of infection no induction of CpcA expression occurs as validated by a GFP-CpcA reporter strain.

Resulting from these data a deeper insight into the proteome depending on the basal expression level of CpcA was sought. Therefore, DIGE experiments were performed to compare the *wild-type* with the *ΔcpcA* strain under non-starvation conditions, which resulted in identification of 22 different proteins. 14 of them displayed an increased level in the *wild-type* in comparison to the deletion strain. Functional classification pointed out that three of the up-regulated proteins might play a role in pathogenicity; in addition, one protein was identified that appears to be required for detoxification of reactive oxidative species.

Accordingly, this study characterises the Cross-Pathway Control system of *A. fumigatus* as a wide-domain regulatory system of this human pathogen.

Zusammenfassung

In dieser Arbeit wurde die Cross-Pathway Control (CPC) des opportunistisch pathogenen Pilzes *A. fumigatus* im Hinblick auf mögliche Faktoren untersucht, die eine Anpassung des Organismus innerhalb des Wirtes ermöglichen. Aus vorherigen Studien war bereits bekannt, dass der transkriptionelle Regulator CpcA dieses Systems Einfluss auf die Virulenz hat.

Im ersten Teil dieser Arbeit wurden mittels Transkriptomanalyse der Wildtyp und eine *cpcA*-Deletionsmutante unter Aminosäuremangelbedingungen miteinander verglichen. Dabei wurden 377 Gene identifiziert, deren Expression unter diesen Bedingungen induziert wurde, während die Expression von 146 Genen reprimiert wurde. Die Klassifizierung dieser Gene zeigte, dass die Mehrheit in Wege des primären und sekundären Metabolismus involviert ist. Darüber hinaus wurden eine Vielzahl an Transportern und Permeasen sowie Transkriptionsfaktoren gefunden, als auch ein Dutzend relevanter Gene bzgl. Stressantworten wie z. B. der Neutralisierung oxidativer Moleküle oder Resistenzmechanismen. Unter anderem wurden dabei zwei sog. AAA-ATPasen entdeckt, die aufgrund der Tatsache, dass sie Teil des CpcA-abhängigen Transkriptoms sind, als *cdaA* und *cdaB* bezeichnet wurden. Entsprechende Mutantenstämme zeigten als Phänotyp eine ausgeprägte Sensitivität gegenüber dem Zellwandstress induzierenden Wirkstoff Calcofluor White. Diese Ergebnisse zeigen schließlich die Anpassungsfähigkeit des Pilzes auf unterschiedliche Stresseinflüsse mittels des transkriptionellen Regulators CpcA.

Das zweite Projekt beinhaltete die Untersuchung der Sensor Kinase CpcC und deren Einfluss auf die Pathogenität von *A. fumigatus*. Die hierfür konstruierte Mutante zeigte unter Mangelbedingungen nur eine begrenzte Induktion von CpcA im Vergleich zum Wildtyp, was zu einem verminderten Wachstum unter Aminosäuremangelbedingungen führte. Trotz dieser geringeren Menge an CpcA zeigte der CpcC Deletionsstamm keine verringerte Virulenz im Mausmodel. Daraus wurde die Schlussfolgerung gezogen, daß die basale Expressionsrate von CpcA ausreichend für die Pathogenität von *A. fumigatus* ist. Dies wird durch die Tatsache unterstützt, dass während der frühen Infektionsphase keine Induktion der CpcA-Expression stattfindet, wie mit Hilfe eines *gfp::cpcA* Reporterstammes festgestellt werden konnte.

Entsprechend dieser Ergebnisse war es das Ziel, das vom basalen Expressionslevel abhängige Proteome zu charakterisieren. Dieses wurde mittels DIGE-Experimenten untersucht, was zur Identifizierung von 22 Proteinen führte, von denen 14 in einer *ΔcpcA*-Mutante eine verminderte Expression aufwiesen. Die weitere Klassifizierung in Funktionsgruppen zeigte, dass drei dieser 14 Proteine gegebenenfalls eine Rolle für die Pathogenität von *A. fumigatus*

spielen. Des Weiteren wurde ein Kandidatenprotein gefunden, dessen Funktion in der Neutralisierung von oxidativen Substanzen liegt.

Diese Arbeiten charakterisieren dementsprechend das Netzwerk der Cross-Pathway Control des Humanpathogens *A. fumigatus* als umfassendes regulatorisches System.

Chapter 1

Introduction

1.1 Life and characteristics of *Aspergillus fumigatus*

The filamentous fungus *Aspergillus fumigatus* belongs to the phylogenetic group of the Deuteromycota, a subgroup of the Euascomycota. As a saprophytic microorganism it is able to colonise and grow on decomposing substrates, making the soil and places like hay or compost its natural habitat where it plays an important role in recycling nitrogen and carbon (Debeaupuis *et al.*, 1997; Mullins *et al.*, 1976). To grow on these materials, *A. fumigatus* secretes degrading enzymes to digest them into oligomeric or monomeric units, which then can be taken up by the fungal cell.

As it is typical for a Deuteromycete, a sexual cycle has not been described yet for *A. fumigatus*. During its asexual lifecycle, *A. fumigatus* produces small spores as it is typical for aspergilli, which are grey-green in colour and have a size of 2 to 3 μm. These conidia contain each one haploid nucleus and are developed from specialised cells called phialides, which are part of the spore-producing structures, the conidiophores. These extend from so-called foot cells at right angles to the vegetative mycelium to culminate in a vesicle with a size of 20-30 μm in diameter. In general, the upper parts of aspergilli conidiophores are built up by layers of specialised cells such as metulae, phialides, and conidia (fig. 1.1), with *A. fumigatus* lacking metula cells. To spread out its hydrophobic spores into the environment, the fungus depends on air currents as it lacks active mechanisms of spore dispersal (Brakhage and Langfelder, 2002; Latgé, 1999). When a spore reaches a place rich in nutrients, germination is triggered to initiate the vegetative growth state. In this state, *A. fumigatus* produces a white-grey mycelium consisting of branching hyphae. These are septated and contain multiple nuclei per cell. Growth and mycelial extension is executed at the hyphal tip to form a dense netting of intertwined hyphae. After a certain time, conidiophores can be formed from this mycelium to produce new spores. These conidia are then spread into the environment and a new cycle begins (fig. 1.2).

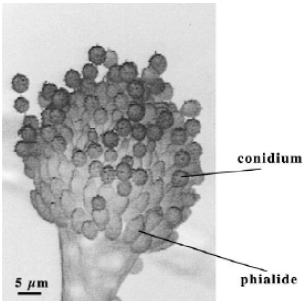

Figure 1.1. Electroscope microscopy image of a conidophore of *A. fumigatus* Shown is the asexual fruit body, the conidiophore, which is built up by vesicles and phialides, from which conidia are formed in chains by repeated budding (from Brakhage and Langfelder, 2002).

A. fumigatus is a fast growing fungus able to reach a colony size of 4 cm in a week (Raper and Fennel, 1965). Its optimal growth temperature is 37°C, but it is able to grow at higher or lower temperatures. In general, fungi can be classified into thermophilic and thermotolerant species based on their minimum and maximum growth temperatures. In this regard, *A. fumigatus* is a thermotolerant fungus able to grow from under 20°C up to 55°C, which is in contrast to thermophilic ones with a growth temperature minimum at or above 20°C and a maximum at or above 50°C. This is an important characteristic that distinguishes *A. fumigatu*s from other aspergilli like *A. flavus*, *A. niger*, or *A. terreus* (Chang *et al.*, 2004; Cooney and Emerson, 1964; Maheshwari *et al*., 2000).

As mentioned above, *A. fumigatus* lacks an obvious sexual cycle. However, in earlier studies it was shown that two different mating-types exist for this Deuteromycete, which leads to the suggestion that a sexual cycle is possible (Dyer and Paoletti, 2005). It is known that parasexuality, which is typical for asexual fungi and enables genetic recombination to a certain degree, is present in *A. fumigatus*. The first step of the parasexual cycle is that different *A. fumigatus* strains grow next to each other, followed by hyphal fusion events and plasmogamy to form a stabilised heterokaryon containing nuclei of both parental strains. Within this, infrequent diploidisation and haploidisation events result in chromosome exchanges and therefore recombination of the genetic contents (fig. 1.2). As a result, a mycelium is formed that enables the fungus to develop a selective advantage in its natural environment (Berg and Garber, 1962; Stroemnaes and Garber, 1962).

With the completed sequencing of *A. fumigatus* in 2005 the research on this organism became easier resulting in a better understanding of this filamentous fungus. On eight chromosomes 9,926 putative protein encoding genes were found with a total size of 29.4 Mb. Nearly a third of the predicted genes are of unknown function (Nierman *et al.*, 2005; Ronning *et al.*, 2005). Thus more time will be required for a final annotation of the *A. fumigatus* genome.

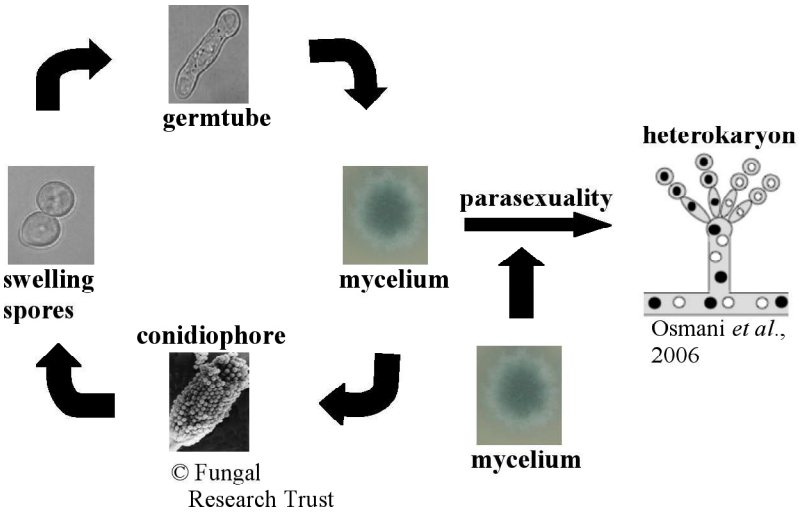

Figure 1.2. Lifecycle of the filamentous fungus *A. fumigatus*
The scheme displays the asexual cycle of *A. fumigatus* with its possibility for parasexuality with a different strain resulting in a stabilised heterokaryon.

1.2 One fungus - three diseases: *Aspergillus fumigatus* & aspergillosis

As outlined above, *A. fumigatus* belongs to the group of saprophytic, filamentous fungi dwelling the soil as a natural habitat. Moreover, this fungus can also be an opportunistic pathogen for humans. In the last two decades, *A. fumigatus* has become one of the most dangerous fungal pathogens for immunocompromised individuals, with the risk of acquiring a disease termed aspergillosis that is usually localised in the respiratory tract. The primary site of entry is the host's lung, although infection by *A. fumigatus* is not limited to this location as it can colonise the skin or disseminate to other organs like kidneys and liver (Denning, 1998; Dixon and Walsh, 1992; Kwon-Chung and Bennett, 1992; Latgé, 1999; Lortholary *et al*., 1995; Prescott *et al.*, 1992). Primarily, infection results from inhalation of the airborne spores, which are small enough to reach the aveoli of the respiratory system. Most patients suffering from aspergillosis have an impaired immune system that is often evoked by leukaemia, neutropenia or after prolonged treatment with steroids, such as solid organ transplantation patients. The mortality rate of aspergillosis among these patients lies between 30 to 90% (Ellis, 1999; Latgé, 1999). To a limited extent, immunocompetent persons can acquire aspergillosis, but the number of incidences is by far not as high as compared to the one among immunocompromised patients (Ellis, 1999).

1.2.1 The different types of disease

Aspergillosis can be divided in three different forms of disease: allergic bronchopulmonary aspergillosis (ABPA), the so-called aspergilloma, and invasive forms of aspergillosis (IA) (fig. 1.3).

ABPA is one of the most severe allergic pulmonary disorders that is induced by aspergilli. It resembles classic asthma with an immune reaction of T-cells against the fungus (Latgé, 1999; Murali *et al.*, 1997; Patterson *et al.*, 1986). This kind of aspergillosis is predominantly found in patients suffering from asthma or cystic fibrosis and may result in a fatal destruction of the lung (Patterson *et al.*, 1982; Rosenberg *et al.*, 1977). In weaker forms it appears as an asthmatic illness without severe damage of the respiratory tract. One major problem is still to diagnose this kind of disease and to distinguish between usual forms of asthma where no fungus is involved and the *Aspergillus*-depended form. The standard diagnostic techniques differ from patient to patient as well as from the status of the disease. Means of ABPA diagnosis are still limited, which often results in undetectable disease leading to the concept of "silent" ABPA (Schönheyder *et al*., 1988). In most cases, patients having an ABPA have to be treated with antifungal drugs; in the worst case scenario, untreated patients may die by the failure of the respiratory system (Latgé, 1999).

The second form of aspergillosis is the so called aspergilloma, often referred to as "fungus ball" (Shibuya *et al.*, 2006). Typical patients are persons having a cystic disease of the lung or a tuberculosis infection. The aspergilloma is usually a limited ball of mycelium without invasive growth. In some cases the non-invasive proliferation of hyphae turns into an invasive form of aspergillosis, e.g. when the immune system is impaired by suppressive therapies or by some kind of illness (Shibuya *et al.*, 2006). In immunocompetent persons the fungus has not the ability to grow invasively, a dissemination of invasive aspergillosis is therefore unlikely.

The third type of aspergillosis is the so-called invasive type (IA), which is the most severe form of disease evoked by this fungus. Mortality rates in this case range from 30 to 90%, and this disease is characterised by invasive fungal growth within the host. IA can be sub-divided in four types which are (Denning, 1998; Hope *et al.*, 2005; Verweij and Denning, 1997): 1.) acute or chronic pulmonary aspergillosis (Latgé, 1999); 2.) tracheobronchitis and obstructive bronchial disease with various degrees of invasion of the mucosa and cartilage as well as pseudomembrane formation, as it is predominantly found in AIDS patients (Denning *et al*., 1991; Kemper *et al.*, 1993; Nash *et al.*, 1997); 3.) acute invasive rhinosinusitis (Drakos et al., 1993; Morgan *et al*., 1984; Savage *et al*., 1997; Viollier *et al.*, 1986; Washburn and Bennett *et al*., 1988); 4.) disseminated disease founding in the brain and other organs of the host (Bodey

et al., 1992; Pagano *et al.*, 1996; Ribaud *et al.*, 1999; Wingard *et al.*, 1987). In the early state of IA it is very difficult to diagnose an infection by common techniques like CT scan or microscopy. Symptoms giving a hint for an invasive aspergillosis are often too unspecific. Therefore, an improved diagnostic regime is needed in order to detect the different forms of IAs (Latgé, 1999).

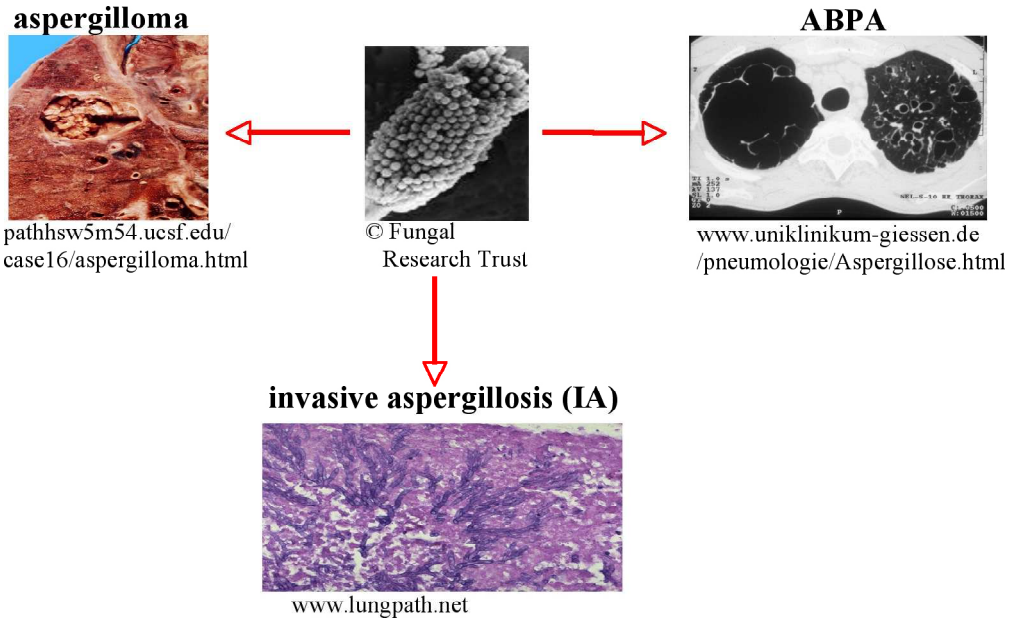

Figure 1.3. The three forms of aspergillosis
The figure displays the three typical types of aspergillosis evoked by different aspergilli like *A. fumigatus*. The aspergilloma and the ABPA (allergic bronchopulmonary aspergillosis) are in general no invasive forms, in contrast to the IA, the worst form of aspergillosis.

1.2.2 The immune-defence-system and therapies against aspergillosis

Invasive forms of aspergillosis normally appear in persons, whose immune system is suppressed by some kind of illness such as leukaemia or by treatment with pharmaceuticals after organ transplantations. In a functional immune system there are different defence mechanisms to react on invasive growth of parasitical organisms. One important group are cells that have the ability for phagocytosis (Latgé, 1999). On one hand there are macrophages, which are the first defence barrier of the host against the fungus. These cells have the ability to bind *via* lectin-like interactions to a special receptor, which can be found on the cell wall of the conidia (Kan and Bennett, 1988, 1991; Serrano-Gomez *et al.*, 2004). After absorption, the spore resides in the phagosome where lysis starts. The dying process of the spores in macrophages is slow, so that after 24 hours still 10% of the ingested spores survive (Schaffner, 1985; Schaffner, 1992). The second phagocytosing barrier is constituted by neutrophils. These are specialised on germinating spores and single hyphae emerging from them. Mechanisms for killing spores are based on destroying the fungal cell wall. For

recognition of the germinating conidia neutrophils detect the same cell wall protein as macrophages do (Serrano-Gomez *et al.*, 2004). Killing by neutrophils happens fast, so that after 2 hours 50% of the phagocytised spores are eliminated (Roilides *et al*., 1991). Additionally, T-cells are activated and seem to be an important defence against invasion (Hohl and Feldmesser, 2007). In an immune-suppressed individual with impaired function or absence of macrophages and/or neutrophils, fungal spores will not be attacked and can germinate without any restriction by the host. As also the mycelium cannot be attacked, the fungus gets the ability to grow invasively and may reach the blood stream from which other parts and organs of the host can be infected.

To date, there are four drugs that are used generally against aspergillosis (Espinel-Ingroff *et al.*, 2005). One of them is amphotericin B (amB), still the "gold standard" against this disease evoked by *A. fumigatus*. This drug binds to membrane sterols resulting in the formation of membrane channels, which increase the cell's permeability. Moreover, it inhibits the activity of proton ATPase pumps. There are two main disadvantages of amphotericin B: one is its insolubility in water and therefore difficulties in applying the drug to the host at sufficient doses; the other point is its toxicity, based on the fact that amphotericin B, although being a reagent that targets ergosterol, displays a pronounced affinity to the cholesterol of mammalian cells, resulting in their destruction. Nevertheless, to date amphotericin B is the most effective antifungal drug against *A. fumigatus* (Bolard, 1986; Brajtburg and Bollard, 1996; Clements and Peacock, 1990; Meletiadis *et al.*, 2007; Pathak *et al*., 1998; Patterson, 1998). Other common anti-fungal drugs are itraconazole, voriconazole and posaconazole, which belong to the triazole family. The effect of the triazoles in general lies in inhibition of ergosterol biosynthesis. Itraconazole, for example, inhibits the enzyme cytochrome P-450 14α-demethylase, which is important for ergosterol production. Reduced levels of ergosterol increase membrane fluidities and thus result in increased permeability. One of the advantages when using triazoles as antifungal drug is that these are very specific for fungi and have less effect on mammalian cholesterol biosynthesis. Thus, there are less adverse effects in IA patients and the compatibility is higher as it is with amphotericin B. However, triazole-containing drugs bear some disadvantages: It was shown that some *A. fumigatus* strains acquires resistance especially against itraconazole; also an i.v. preparation for itraconazole is not available (Chryssanthou, 1997; Denning *et al*., 1997; Denning *et al*., 1997; Latgé, 1999). This problem does not exist for other triazoles like voriconazole, which have also other advantages in comparison to itraconazole (Pfaller *et al.*, 2002). A common drawback is the high amount of required drug, but the concentration shows no strong adverse effects in

patients with invasive aspergillosis. Nevertheless, all of these used triazoles show less effect against *A. fumigatus* than amphotericin B and are mostly fungistatic in contrast to amB, which is fungicidal. The effects of the described antifungal drugs are typical for *A. fumigatus* and may differ for different species of aspergilli such as *A. flavus* or *A. terreus* (Meletiadis *et al.*, 2007). In general it has to be stated that the palette of different antifungal drugs is still limited and new appendages of therapies and pharmaceuticals have urgently to be found.

1.2.3 Virulence determinants of *A. fumigatus*

To invade the host and overcome any residual immune system, *A. fumigatus* needs special abilities (Latgé *et al*., 1997; Latgé *et al*., 1994). It must be able to survive in a nutrient-limited area for which specific biosynthetic pathways might be necessary. One important point to initiate growth inside the host is the production of adhesins, which enable *A. fumigatus* to adhere at the respiratory epithelia. Thereby the conidia are able to bind with their cell surface on membrane-associated proteins. These interactions between the spores' cell wall and host proteins can happen both in a specific or unspecific manner. Typical binding targets for the fungus are fibrinogen, laminin, fibronectin, and type IV collagen (Bromley and Donaldson, 1996; Gil *et al.*, 1996; Penalver *et al.*, 1996; Tronchin *et al.*, 1993). The interacting compounds and molecules for adhesion to proteins of the host epithelium, which can be e.g. carbohydrates, protein molecules and glycoproteins, are localised in the inner and outer cell wall layer. To bind on hydrophobic proteins, *A. fumigatus* contains so-called hydrophobins that are located in the surface layer of dry conidia and can also been found in other fungal spores. Although all these adhesins appear important for fungal adhesion in the host, their role in or contribution to pathogenicity is still unclear (Latgé, 1999; Thau *et al.*, 1994).

Conidial pigmentation, resulting in the typical grey-green colour, seems to be important for fungal resistance. White spores have a more permeable cell wall and are more susceptible to antifungal drugs compared to *wild-type* conidia. An important compound in pigmentation is melanin, which likely enables the fungus to counteract the immune defence system and increases spore resistance (Hogan *et al*., 1996; Latgé, 1999; Tsai *et al.*, 1997; Verweij *et al.*, 1998). Deletion of the *pksP* laccase results in white-coloured spores, which are reduced pathogenicity compared with the wild-type (Jahn *et al.*, 1997; Jahn *et al.*, 2000; Langfelder *et al.*, 1998; Langfelder *et al.*, 2001; Tsai *et al.*, 1998). In contrast to this leads the deletion of the *abr2* gene, which encods also a laccase in *A. fumigatus*, not to a reduction of pathogenicity (Sugareva *et al.*, 2006). To summarize it can be said that proteins involved in pigmentation are not necessarily required for virulence.

Other putative virulence factors of *A. fumigatus* are toxic molecules, which are often products of secondary metabolism. Gliotoxin is one of the best-studied toxins of *A. fumigatus*. It inhibits the phagocytosis by macrophages and can induce their apoptosis. This effect could also be seen for polymorphonuclear leucocytes (PMN) but in a reduced form (Eichner *et al.*, 1986; Kamei and Watanabe, 2005; Orciuolo *et al.*, 2007; Waring *et al.*, 1988). Other molecules with a toxic effect are the 18-kDA RNase and a hemolysin (Asp-HS). The RNase has the ability to digest the 28S rRNA of eukaryotes. The protein is secreted into the environment and can kill cells that are in the vicinity of the spore. In IA patients this secrete can be found in the urine (Arruda *et al.*, 1992a; Arruda *et al.*, 1992b; Kurup *et al.*, 1994; Lamy and Davies, 1991; Latgé, 1999). The hemolysin, which enables the fungus to disrupt blood cells, contains negatively charged domains and can also be detected in infected patients. However, despite of the facts that the hemolysin has toxic effects it seems not to be a main virulence factor but a compound that increases the effects of other toxic factors involved in pathogenicity (Ebina *et al.*, 1983; Fukuchi *et al.*, 1996; Malicev *et al.*, 2007; Yokota *et al.*, 1985). An additional large group of putative virulence factors are enzymes displaying different kinds of function. Some proteases for example seem important for full virulence of *A. fumigatus*. They are needed to obtain nutrients by degradation of collagen and elastin, which constitute the main compounds of the lung (Monod *et al.*, 1995). In further works it could be shown that clinical isolates have a higher production of proteases than the environmental pendants, and some of these enzymes lead to a detachment of epithelium cells in the respiratory tract. The major protease under neutral conditions is the serine alkaline protease *ALP* with the ability to proteolyse elastin. Nevertheless, strains without *ALP* cause the same mortality rate in a mouse model compared to those with *ALP*. Other enzymes without proteolytic activity are also important for *A. fumigatus* in causing disease (Grant, 2001; Smith *et al.*, 1994; Tang *et al.*, 1992; Tang *et al.*, 1993; Tekaia and Latgé, 2005): catalases and peroxidases are needed by the organism to counterattack reactive oxidative species like H_2O_2 (Shibuya *et al.*, 2006). Deletion of four enzymes which are involved in peroxide breakdown lead to strains which are more sensitive to this reactive oxidative molecule but not to phagocytosis (Paris *et al.*, 2003). Thus, the role of catalases and peroxidases for *A. fumigatus* pathogenicity has still to be proven.

In general it has to be stated that pathogenicity of *A. fumigatus* appears to be multifactorial and cannot be assigned to one single trait (d'Enfert *et al.*, 1996; Latgé, 1999; Tekaia and Latgé, 2005).

1.3 The lung as an environment for *A. fumigatus* with nutrient limitation

As described before, *A. fumigatus* is a typical saprophyte that can degrade decaying material in order to gain nutrients from the environment (Debeaupuis *et al.*, 1997; Mullins *et al.*, 1976). When spores of this fungus colonise the lung of the host, nutrients are likely to be limited. Nitrogen and carbon sources may not be as easy available as it is the case in the natural environment. Therefore, the fungus depends on special metabolic pathways that support its growth under depleted conditions. For other pathogenic organisms it was shown that these kinds of pathways are often involved in pathogenicity. Earlier studies pointed out that the lipid metabolism of pathogenic organisms like *Candida albicans* and *Mycobacterium tuberculosis* is essential for virulence (Bishai, 2000; Lorenz and Fink, 2001, 2002; McKinney *et al.*, 2000; Munoz-Elias and McKinney, 2005). These organisms are able to extract carbon sources from the host *via* the glyoxylate cycle. The key enzyme of this pathway is an isocitrate lyase, whose deletion leads to a reduced virulence of *M. tuberculosis* and *C. albicans* (Lorenz and Fink, 2001; Munoz-Elias and McKinney, 2005). Investigations of the isocitrate lyase of *A. fumigatus* revealed that in this organism the glyoxylate cycle is not important for virulence as an isocitrate lyase mutant has the same virulence in murine model as the wild-type, and this lead to the suggestion that the lipid metabolism is not sufficient to exploit C-sources in the lung (Ibrahim-Granet *et al*., 2008; Schöbel *et al*., 2007). Therefore, *A. fumigatus* uses other pathways and enzymes to metabolise nutrients from the environment. The lung of mammals, consisting mostly of proteins like collagen, is a habitat where nutrients are unbalanced. For degradation of these tissues the fungus needs special enzymes like proteases which results in mobilisation of amino acids, which can be used as sources of nitrogen and carbon (Kogan *et al.*, 2004). Free amino acids are absorbed and metabolised *via* different pathways to support fungal growth. An important route in carbon metabolism is the methylcitrate cycle: the amino acids isoleucine, valine and methionine are converted to propionyl~CoA, which then can be channelled into this patway. Deletion of the methylcitrate synthase, a key enzyme of this metabolic route, results in reduced fungal virulence. One possible explanation for this is that propionyl~CoA cannot be metabolised and accumulates in the fungus where it has a toxic effect (Brock and Buckel, 2004; Ibrahim-Granet *et al.*, 2008; Maerker *et al.*, 2005; Zhang and Keller, 2004). As a result growth in the host lung is reduced. Other metabolic pathways appear to be also important for *A. fumigatus* during invasive growth. Primary metabolism pathways involved in producing substances like para-aminobenzoic acid, pyrimidine, or lysine are also required for the growth in the host and therefore for full virulence. In addition to carbon and nitrogen, the fungus also needs trace

elements such as iron. In contrast to many other pathogens, *A. fumigatus* does not contain a specific mechanism for utilization of host iron sources (Ramanan and Wang, 2000; Ratledge and Dover, 2000; Schrettl *et al.*, 2004). The fungus uses two systems for iron uptake: the reductive iron assimilation system and the siderophore-assisted iron mobilization system (Haas, 2003; Leong and Winkelmann, 1998; Van Ho *et al.*, 2002). In earlier works it was demonstated that the *sidA* gene, which is involved in biosynthesis of an *A. fumigatus* siderophore, is important for growth in the host. In contrast to this, the *ftrA* gene encoding an iron permease that is involved in the iron assimilation system seems not to be important for survival in the host (Schrettl *et al.*, 2004).

To react on limited and changing conditions of the environment, the fungus needs special sensors and regulatory proteins by which uptake systems and metabolic pathways are induced. Changing conditions of glucose for example are detected by the cAMP/protein kinase (Ruijter and Visser 1997). The nitrogen assimilation under poor conditions is regulated by different kind of proteins like the *areA* gene product, the Ras-related protein RhbA, or CpcA, the regulator of the Cross Pathway Control (CPC) system of amino acid biosynthesis (Hensel *et al.*, 1998; Krappmann *et al.*, 2004; Panepinto *et al.*, 2002; Panepinto *et al.*, 2003). Deletion of the *areA* gene leads to delayed invasive growth in the host resulting from the disability to take up certain nitrogen sources. Strains without the *rhbA* gene are also attenuated in virulence like a *cpcA* deletion strains. Integral to the CPC is an eIF2 kinase, which is the sensory element of this system. It can react on different kinds of limitations like amino acid starvation and stress conditions perceived from the environment (Natarajan *et al.*, 2001) of this sensor kinase resulting in increased levels of the transcriptional activator CpcA that in turn activates genes important for different kind of metabolic pathways, among them cellular amino acid biosynthesis.

1.4 The Fungal Cross-Pathway Control System

Microorganisms have to be able to react to changing environmental conditions. In order to have a higher chance of survival under rapidly evolving environmental situations, they must have a system that is sensitive and that enables them to react on these ascendancies. Such kinds of regulatory systems were detected in various fungi like the yeast *Saccharomyces cerevisisae* (Hinnebusch, 1984) or in filamentous moulds like *Neurospora crassa* and *Aspergillus nidulans* (Carsiotis *et al.*, 1974; Davis, 2000; Piotrowska, 1980; Sachs, 1996) where they were named “General Control of amino acid biosynthesis” (GC) or “Cross-Pathway Control” (CPC), respectively. They are activated under amino acid starvation

conditions and result in a regulation of different sets of genes involved in amino acid biosynthesis, purine biosynthesis, nitrogen and sulfur metabolism and vitamin biosynthesis (Hinnebusch, 1997; Mirande, 1988; Mösch *et al*., 1991; Natarajan *et al.*, 2001; Tian *et al.*, 2007).

In its core, this signal transduction system consists of a sensor kinase, which is able to phosphorylate a subunit of the eukaryotic translation initiation factor eIF2, and a transcriptional activator that serves as downstream effector to generate a cellular read-out.

1.4.1 Induction and regulation of the CPC/GC

The regulatory gene of the yeast General Control was named *GCN4* (*g*eneral *c*ontrol *n*on-derepressible), and its mRNA contains four small coding sequences, which are part of an unusually long leader region preceding the actual coding sequence. The *Aspergillus* gene homologous to *GCN4* is *cpcA*. The *cpcA* gene of *A. fumigatus* has a size about 2.2 kb, from which the coding sequence spans 810 bp. Two small ORFs exist preceding the *cpcA* coding region, which are similar to the four regulatory uORFS of *GCN4* (fig. 1.4).

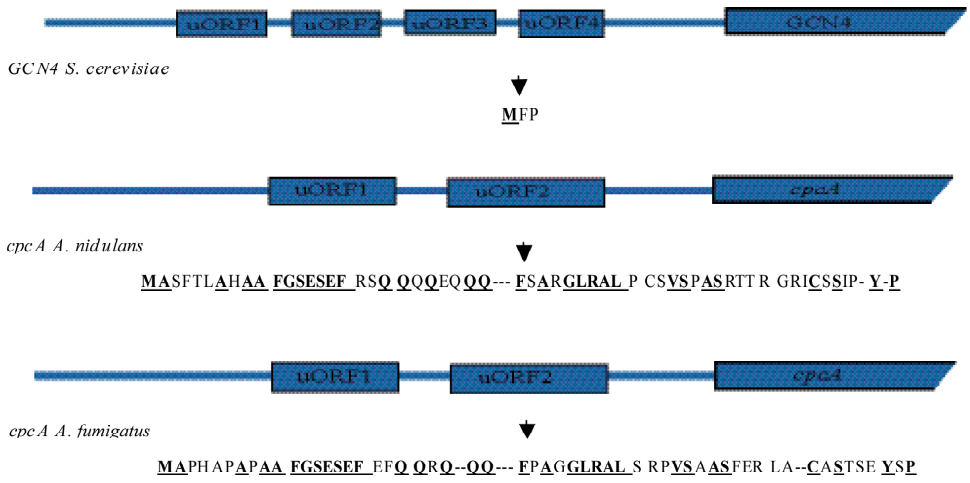

Figure 1.4. Comparison of the *cpcA* in *A. fumigatus* with the *GCN4* of *S. cerevisiae* and the *cpcA* of *A. nidulans*. The figure shows the single uORFs of *cpcA* and *GCN4* respectively. The amino acid sequence of the 4th uORF of *S. cerevisiae* and the 2nd uORFs of *A. nidulans* and *A. fumigatus* is written below the corresponding gene. Homologous parts of the sequences are marked bold and underlined.

The relationship between structure and function of the *GCN4* gene product has been investigated in detail. The C-terminal part contains a leucine-zipper motif that is important for the dimerisation of the protein. Furthermore, a conserved DNA binding region can be found in the C-terminal domain, which is important for transcriptional regulation of Gcn4p target genes (Ellenberger *et al.*, 1992). The N-terminal region is divided in two parts that represent

different transcriptional activation domains (Drysdale *et al.*, 1998). All these elements are conserved in the *A. fumigatus cpcA* gene product as estimated from the deduced amino acid sequence. The CpcA protein consists of 252 amino acids and has a calculated molecular weight of about 27 kDa. The activation of other genes by CpcA is affected by the binding at the promoter regions of these genes resulting in increased levels of transcription.

Regulation of *cpcA* expression in the related fungus *A. nidulans* is mediated on the transcriptional as well as the translational level (Hoffmann *et al.*, 2001), and it is likely that regulation of *A. fumigatus cpcA* resembles this pattern. In contrast to this, expression of *GCN4* in *S. cerevisiae* is mainly regulated on translational level, whereas in *C. albicans* transcription of *GCN4* is more important than translation (Tournu *et al.*, 2005). In detail, translational regulation of gene expression *via* the upstream open reading frames of *GCN4*/*cpcA* transcripts acts like follows: under sated conditions, expression levels are low at a basal level due to the fact that scanning ribosomes initiate translation on the *GCN4/cpcA* transcript at the first uORF. Important for this are the amounts of so-called tertiary complexes, which consist of the translation initiator eIF2, GTP and a $tRNA^{Met}$, and these three components are crucial factors for initiation of translation. The tertiary complex enables the ribosomes to bind at the first uORF within the leader sequence of *GCN4/cpcA*. After reaching the stop codon, ribosomes disassemble into the two ribosomal sub-domains. To initiate another round of translation, a new tertiary complex has to be assembled together with the small ribosomal subunit. In case concentrations of tertiary complexes are high, one of the downstream uORFs will be translated. As the distance between the stop codon of the distal uORF and the start codon of the actual coding sequence is too close translational re-initiation is hampered and almost no gene product is expressed. Under starvation conditions expression is drastically de-repressed. In case of amino acid starvation, uncharged tRNA molecules accumulate and bind to a sensor kinase called Gcn2p or CpcC, respectively. The activated kinase phosphorylates a subunit of the initiation factor eIF2, resulting in diminished levels of tertiary complexes and therefore lower rates of translation initiation. In consequence, overall cellular translation is down-regulated but translation initiation at the *cpcA/GCN4* coding region increases, as competent ribosomes that have failed to translated the distal uORF are now able to bind at the start codon of the coding sequence to initiate the translation (fig. 1.5) (Braus *et al.*, 2004).

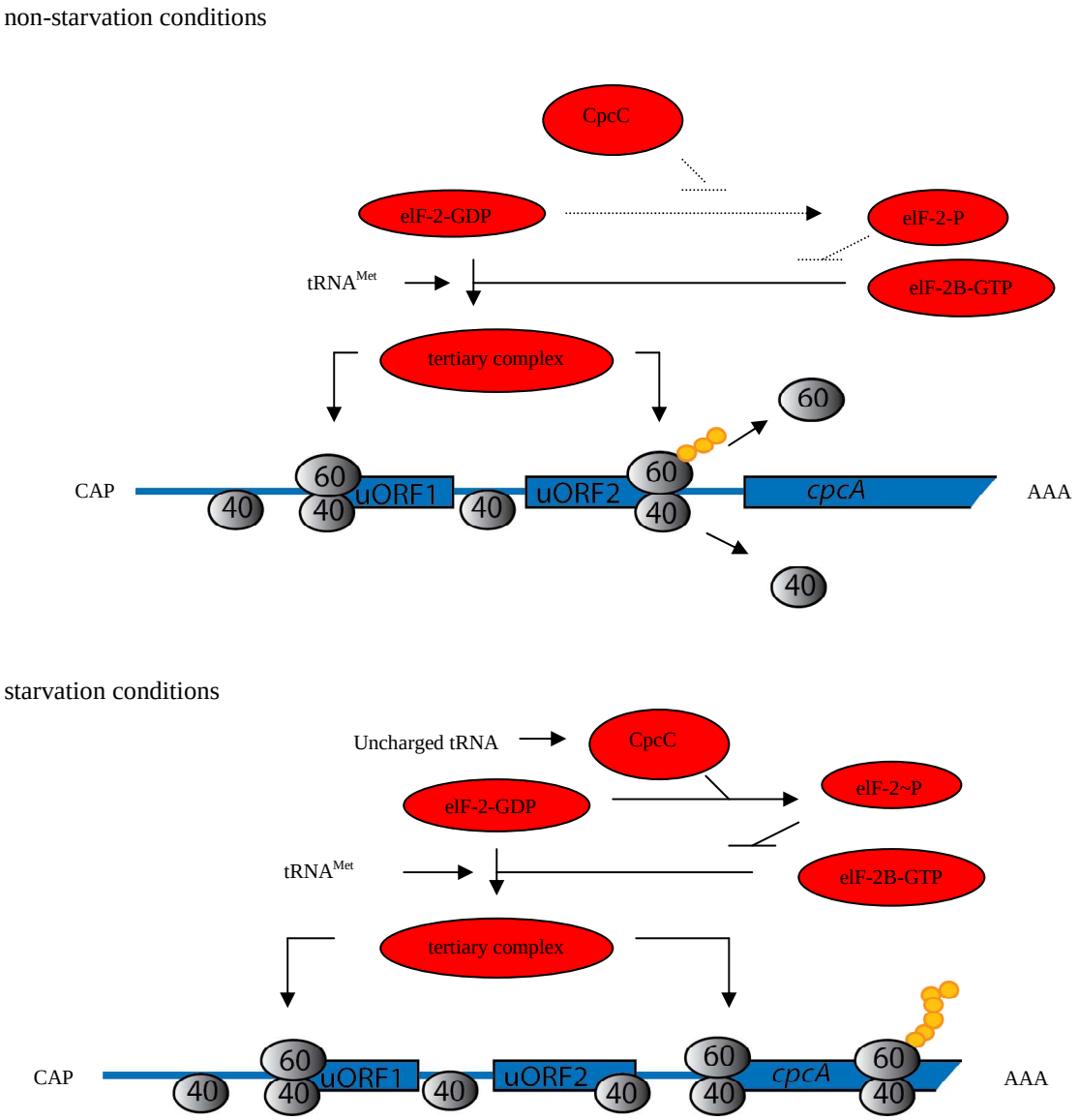

Figure 1.5. Regulation of translation of *cpcA* in *A. fumigatus*
The picture shows a hypothetical model of the regulation of *cpcA* corresponding to the yeast model. Under non-starvation conditions sufficient amounts of charged tRNA molecules are in the cell resulting in high levels of tertiary complexes. This leads to a translation of the 1st and 2nd uORF but not of the coding sequence of the gene. Under starvation conditions the level of uncharged tRNAs increases, which is recognised by the sensor kinase CpcC. Activated CpcC phosphorylates eIF-2-GDP to eIF-2~P, which leads to a lower amount of tertiary complexes. Thus, the coding sequence of the 2nd uORF cannot be translated and the scanning ribosomes bind to the start codon of the *cpcA* coding region to initiate translation.

Regulation of *Aspergillus* CpcA expression on the transcriptional level has been investigated in detail for *A. nidulans* (Hoffmann *et al.*, 2001). There, synthesized CpcA binds to so-called CPREs (*C*ross-*P*athway Control *R*ecognition *E*lements) that locate within the promoter region of target genes. It could be shown that two of these CPREs are part of the promoter region of *cpcA* suggesting a functional auto-regulatory loop (Hoffmann *et al.*, 2001). A regulation like this has not been proven for CpcA expression in *A. fumigatus*; however, it was shown that two

highly conserved CPREs are also present within the *cpcA* promoter region (Krappmann *et al.*, 2004). The proximal one (5'-ATGACTCAC-3', pos. -1062 till -1053) is identical to its *A. nidulans* counterpart, the distal one shows a difference in one site of the sequence in comparison to the *A. nidulans* CPRE (5'-ATGACTCgAC-3', -1273 till -1264). This leads to the suggestion that binding of CpcA to this recognition site might be weaker (Arndt and Fink, 1986; Hinnebusch, 1984; Thireos *et al.*, 1984).

In addition, a variety of other factors that influence activity of the Cross-Pathway Control/General Control activators CpcA/Gcn4p were identified, among them: glucose, nitrogen and purine starvation (Braus *et al.*, 2004; Grundmann *et al.*, 2001; Hinnebusch and Natarajan, 2002) (fig. 1.6).

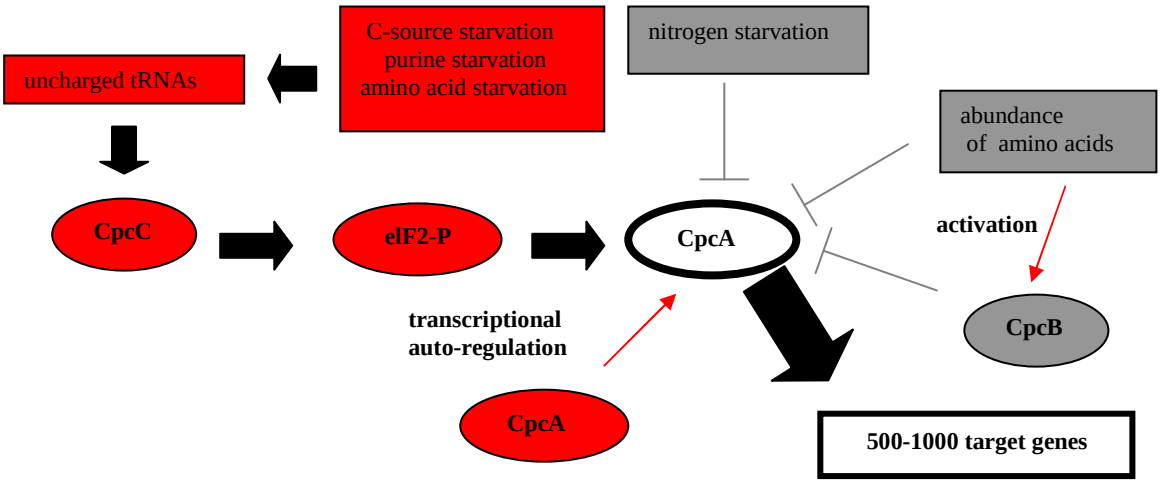

Figure 1.6. Factors with an effect on CpcA
The scheme displays the different kinds of factors that have an effect on CpcA. The arrows show activating influences on CpcA.

1.4.2 CPC & Pathogenicity of *A. fumigatus*

As outlined above, pathogenicity of *A. fumigatus* depends on several attributes, and conclusively, a plethora of genes and their products influence its virulence (Latgé, 2001). In earlier works it was demonstrated that *cpcA* has an influence on pathogenicity of *A. fumigatus* (Krappmann *et al.*, 2004). In this particular study, *cpcA* deletion mutants had been generated lacking either the coding region or the complete gene locus. Both strains showed, in comparison to the *wild-type*, reduced growth on minimal medium containing 5-methyl-DL-tryptophan (5MT), which is a tryptophan analogue and leads to a feedback inhibition of the tryptophan biosynthesis resulting in amino acid starvation conditions. The reconstitution of both strains with *cpcA* led to normal growth on 5MT containing medium. In histidine-

depleted cultures, both deletion strains showed no induction of the Cross-Pathway Control, as it was shown in Northern experiments using qualified reporter genes. The influence of *cpcA* with respect to pathogenicity was tested in a murine model of pulmonary aspergillosis using leukopenic mice. Infection of the mice with the *wild-type* strain resulted in a dying rate of 85% after five days. In contrast to this, dying rates among the group of mice infected with the *cpcA* deletion strains were at 44% and 31% after 5-6 days. Homologous reconstitution of the *cpcA* locus in the deletion strains restored pathogenicity to *wild-type* levels. Furthermore it could be shown by competitive infection in immunosuppressed mice that the wildtype had a growth advantage in comparison to either *cpcA* deletion strain. These results demonstrate that the absence of the *cpcA* gene product results in attenuated virulence. One possible explanation for this phenotype is nutritional limitation within the host's lung, making the Cross-Pathway Control system necessary for fungal growth and therefore virulence.

1.5 Aim of this work

In recent decades, *A. fumigatus* has become one of the most menacing pathogenic fungi for immunocompromised individuals. Factors and characteristics that enable the fungus to change from a saprophyte to a pathogen are still unclear. It was shown that special mechanisms and pathways contribute to virulence and that pathogenicity of *Aspergillus* is a multifactorial phenomenom (Latgé, 1999). Among these factors is the Cross-Pathway Control system effector CpcA (Krappmann *et al.*, 2004). In this work the Cross-Pathway Control should be analysed with respect to stress resistance and pathogenicity of *A. fumigatus*.

The first project describes the analysis gene expression under conditions of amino acid starvation by means of microarray hybridisations with the aim to get a comprehensive overview on the CpcA-directed transcriptome of *A. fumigatus*. Data from other fungi had revealed that the CPC system is not restricted to conditions of amino acid starvation but may act as a general response system to act upon conditions of nutritional or environmental stress. Therefore, transcriptional profiling should evaluate the *A. fumigatus* CPC system with respect to stress resistance of this pathogen.

The second part of this PhD work deals with the eIF2 kinase CpcC, the orthologue of yeast Gcn2p, and the role of the Cross-Pathway Control during phagocytosis by macrophages. For that purpose a suitable reporter strain had to be constructed, and the *cpcC* gene had to be identified and comprehensively characterised to result in deletion mutant strains. Accordingly, the impact of CpcC in virulence was assessed.

The basal expression level of the Cross-Pathway Control activator CpcA is the main focus of the last chapter. Based on the finding that *cpcC* deletion strains show no difference in virulence to their *wild-type* progenitor suggests that the basal expression level of CpcA is necessary but also sufficient to support pulmonary aspergillosis. Conclusively, gene products depending in their expression on basal expression levels of CpcA should be identified by proteome analyses.

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Chapter 2

CpcA - a Master Regulator of Stress Response Factors

2.1 Abstract

The opportunistic pathogen *A. fumigatus* requires a variety of metabolic pathways and regulatory circuits to grow and survive in the lungs of infected hosts. One of these is the Cross-Pathway Control (CPC) system, which is homologous to the yeast general control (GC) of amino acid biosynthesis. Based on the observation that the transcriptional activator of the *A. fumigatus* CPC, CpcA, is required for full virulence of this fungus, we wanted to find putative CPC-regulated stress genes that are required for infection and survival under stress conditions. In transcriptional profiling studies under amino acid starvation conditions 523 genes were detected that are regulated by CpcA, from which 377 are up-regulated and 146 are repressed. Many of these genes are involved in metabolic pathways like amino acid biosynthesis or sulfur, nitrogen, and carbon metabolism. A large group of permeases and multi-drug transporters as well as transcriptional regulators are also targets of CpcA. Accordingly, these data demonstrate a wide domain regulation executed by the CPC. In addition we detected twelve genes putatively involved in stress response. Two CpcA-dependent AAA-ATPases were also present in the data set, which were designated *cdaA* and *cdaB*. Single and double knock-out mutant strains showed reduced growth on Calcofluor White-containing medium, therefore the corresponding gene products appear to be involved in counteracting cell wall and septa stress. Furthermore, Calcofluor White treatment of *A. fumigatus* resulted in a weak induction of *cpcA* transcription but not to an activation of the Cross-Pathway Control system. These studies support the role of the CPC as a global regulatory system evolved in fungi that is active under diverse environmental conditions of stress.

2.2 Introduction

In the last decade the Deuteromycete *Aspergillus fumigatus* has become one of the most important opportunistic pathogens for immunocompromised hosts such as leukemia patients or recipients of tranplanic organs, which can suffer severely from invasive forms of aspergillosis (Latgé, 1999). The number of infected patients has increased rapidly in the last years with high mortality rates between 30% to 90%, but until now no effective and reliable therapy against this fungus has been developed (Ellis, 1999). A large number of clinical isolates show resistance to established mycostatical drugs, like the standard triazoles such as itraconazole and voriconazole (Espinel-Ingroff *et al.*, 2005). Other antimycotica, like amphotericine B, that have a higher efficiency and to which less strains show a resistance, may be also toxic for the host itself (Denning *et al*., 1997; Denning *et al*., 1997; Meletiadis *et al.*, 2007). Moreover, it is still difficult to diagnose aspergillosis in an early stadium of infection, when a therapy by antifungal pharmaceuticals could lead to a more successful treatment. Therefore, there is still a necessity to find new mycobiotica and therapies that are effective and show no toxicity for the patients. Accordingly, it is important to gain better understanding of *A. fumigatus* pathogenicity in general and a more detailed picture about factors, pathways, and regulatory systems that are involved in virulence of this saprophytic fungus.

In its natural habitat, which is the soil of decaying organic material like compost, *A. fumigatus* is able to react on changing growth and stress conditions (Latgé, 1999; Millner *et al.*, 1977; van Heerden *et al*., 2002). It is also flexible with respect to different temperature ranges, demonstrated by its capacity to grow at levels up to 52-55°C, whereas other aspergilli like *A. flavus*, *A. niger* and *A. terreus* have not this kind of ability (Chang *et al.*, 2004). Therefore, the body temperature of mammalians does not represent a barrier for this fungus and has no negative influence on the colonisation by the spores of the fungus and the germination within the lung tissue. In order to respond to and to counter naturally occurring environmental stress situations like starvation conditions (Hensel *et al.*, 1998; Krappmann *et al.*, 2004; Panepinto *et al.*, 2002; Panepinto *et al.*, 2003; Ruijter and Visser, 1997), changing temperatures, and escalating pH values several signalling cascades and distinct cellular pathways are required. The mammalian lung may represent an ecological niche where starvation conditions exist and nutrients are limited; accordingly, the pathways important for survival and adaptation in the natural *A. fumigatus* habitat may also play a role for germination in the host and therefore as a consequence of invasive aspergillosis. One of these pathways required for adaptation to environmental stress in the natural habitat is the Cross-Pathway Control system (CPC), which

is homologous to the General Control (GC) of amino acid biosynthesis in *S. cerevisisae* (Delforge *et al.*, 1975; Hinnebusch, 2005; Krappmann *et al.*, 2004; Schurch *et al.*, 1974; Wolfner *et al.*, 1975). The CPC system consists of two main components, CpcA and CpcC. The encoding *cpcA* gene is orthologous to yeast *GCN4*, which codes for a transcriptional regulator that acts on a plethora of genes. *cpcC* encodes a sensor kinase that is important for translational regulation of *cpcA* expression in accordance to the yeast mechanism of regulation (Hinnebusch, 2005; Hoffmann *et al.*, 2001). Under sated conditions, two short open reading frames (uORFs) in the upstream region of the *cpcA* gene repress translation of the *cpcA* that results in low amounts of CpcA, the basal expression level. Under environmental stress conditions like amino acid starvation, the CPC sensor kinase CpcC becomes activated to phosphorylate a subunit of the eukaryotic initiation factor of translation eIF2. This results in an increased translation of the transcriptional activator CpcA. Now CpcA can modulate the expression pattern of its target genes enabling the fungus to react to the starvation conditions. In recent studies the yeast general control response under amino acid starvation conditions has been analysed (Hinnebusch, 2005; Natarajan *et al.*, 2001). It was shown that Gcn4p activates more than 30 genes involved in amino acid biosynthesis in twelve different biosynthetic pathways; around 1000 genes are regulated directly and indirectly by this transcriptional activator under these starvation conditions. Furthermore, the transcriptional response of the Ascomycete *Neurospora crassa* under amino acid starvation conditions shows de-repressed expression of genes involved in amino acid biosynthesis, which represents the largest group of genes in the according data set (Tian *et al.*, 2007). Genes with functions in carbon metabolism and nitrogen metabolism were also found to be regulated as well as others, which are involved in the oxidative stress response. In total, the CPC-dependent transcriptome of this filamentous fungus shows a regulation of 443 genes. These data sets give a hint on the importance of the fungal CPC network in order to react to different kinds of environmental stress.

In this study the CpcA-dependent transcriptome of *A. fumigatus* under amino acid starvation conditions was investigated. In earlier works it had been shown that the transcriptional factor CpcA is involved in pathogenicity of *A. fumigatus*: a deletion of the encoding gene resulted in attenuated virulence in a murine model of pulmonary aspergillosis (Krappmann *et al.*, 2004). Therefore, the main focus of the data set was set on putative stress factors regulated by CpcA, which maybe support growth of *A. fumigatus* in the host. Approximately 500 genes were identified to be regulated by CpcA, among them nearly 400 that are influenced in a positive manner. These genes were categorized into different functional groups, such as carbon

metabolism, amino acid biosynthesis, transporters, which represents the largest group of up-regulated genes in the data set, and putative stress genes. Furthermore, the CPC transcriptome was compared with transcriptional profiling data of *A. fumigatus* propagated in the presence of the antifungal drug voriconazole (da Silva Ferreira *et al.*, 2006).

2.3 Materials and Methods

2.3.1 Strains and media

For general cloning procedures the *Escherichia coli* strain DH5a [F–,f80dD(*lacZ*)M15-1, D(*lacZYA-argF*)U169, *recA1*, *endA1*,*hsdR17* (*rK*–, *mK*+), *supE44*, l–, *thi1*, *gyrA96*, *relA1*] (Woodcock *et al.*, 1989) was used. *E. coli* cultures were grown in LB or LBLS (1%bacto-tryptone, 0.5% yeast extract, 1% or 0.5% NaCl, pH 7.5) medium at 37°C. Fungal strains used in this study are listed in table 2.1 Growth of *A. fumigatus* strains was carried out at 37°C on minimal medium prepared and supplemented according to Käfer (1977). Supplemented antibiotics and drugs were ampicillin (100 µg/ml), kanamycin (50 µg/ml), hygromycin B (200 µg/ml), phleomycin (40 µg/ml), pyrithiamine (100 µg/ml).

2.3.2 *A. fumigatus* transformation and plasmid construction

Aspergillus DNA was extracted and isolated according to the protocol of Kolar *et al*. (1988). For genomic analysing Southern hybridisation method was used (Southern, 1975), the probe labelling was done with the DNA labelling kit of GE Healthcare (Buckinghamshire, UK) was used according to the manufacturer's protocol. For DNA amplification Phusion polymerase of FINNZYMES was used as described previously (Saiki *et al.*, 1985).

E. coli transformation was performed according to the protocol of Higa and Mandel (1970). *Aspergillus* strains were transformed by using the polyethylene glycol-mediated fusion of protoplasts (Punt and van den Hondel, 1992).

For constructing the *cdaA* knock out cassette the 5' UTR of the gene was amplified by using the primer pair CS49/CS50 (3.4 kb). The 3' region was amplified by using the primers CS51/CS52 (2.7 kb). The hygromycin resistance cassette was received by digesting pME3002 with *Sfi*I (3.5 kb). All three received fragments were ligated *via* overlapping *Sfi*I sites using a T4 ligase receiving a 5'-marker-3' fragment and transformed in the pCR®-BluntII-TOPO® vector (INVITROGEN GMBH, Karlsruhe, D) according to the manufacturer's protocol. For construction of the *cdaB* deletion cassette the flanking regions of *cdaB* were amplified by using the primers CS45/CS46 for the 5' UTR (2 kb) and CS47/CS48 for the 3' region (2.1 kb). A phleomycin resistance cassette was received by digesting pME2891 with *Sfi*I (2.9 kb). *Via* ligation of the three fragments the construct 5'-marker-3' was received and finally cloned in pBlueskript II KS *via Eco*RV (pCS8). Used primers and plasmids are listed in table 2.2 and 2.3 To delete *cdaA* pCS5 (pCR®-BluntII-TOPO® vector containing the *cdaA* deletion cassette) was digested with *Fsp*I and *Stu*I to receive an 8.9 kb fragment 5'-marker-3', which was transformed *via* homologous integration in the clinical isolate D141 (AfS00). pCS8

(pBlueskript II KS containing the *cdaB* deletion cassette) was digested with *Hpa*I and *Nhe*I receiving a 6.9 kb fragment, which was transformed *via* homologous recombination in the *wild-type* strain D141(AfS00). For construction of the double knock out strain the *cdaB* deletion construct was integrated *via* homologous recombination in the *cdaA* deletion strain. For all polymerase chain reactions genomic DNA was used as template. For marker recycling the protocol was used according to Krappmann *et al*. (2005) with the modification to select transformants on minimal medium containing 25 µM FUDR.

2.3.3 Growth tests

2000 spores in 2 µl of each strain were plated on minimal medium (MM) and incubated for two to three days at 37°C. For amino acid starvation condition 2 mM L-methione sulfoximine (MSX) were used. 1 mg/ml Calcofluor White (SIGMA) containing medium was used to induce stress conditions to cell wall and septa building.

2.3.4 Transcriptome analysis and Northern hybridisation

$2x10^6$ spores per millilitre of the *wild-type* strain D141 and the *cpcA* deletion strain AfS01 were inoculated in liquid MM containing ammonium as nitrogen source. After 17 h incubation at 37°C the cultures were shifted for additional 4 h to fresh MM in the presence or absence of 10 mM 3-amino-1,2,4-triazol (3AT) to evoke amino acid starvation conditions. Mycelia were harvested by filtration with miracloth filter (CALBIOCHEM) and washed thoroughly with sterile water. Total RNA was isolated from liquid cultures by using Trizol (INVITROGEN). 20 µg RNA from each sample were fractionated in formaldehyde, 1,4% agarose gel, stained with ethidium bromide and the visualised with UV-light. The presence of clear ribosomal bands was used as a criterion for good quality. Each RNA sample was digested with DNAse and purified with RNeasy columns from Qiagen and eluted in 70 µl RNAse free water. For hybridisation microarray chips for *A. fumigatus* Af293 from TIGR were used. Hybridisation was accomplished according to the protocol of da Silva Ferreira *et al*. (2006). For verification of the transcriptome data and induction under Calcofluor White conditions Northern experiments according to the protocols cited by Brown and Mackey (1997) were done. Random primed labelling was performed with the STRATAGENE Prime-It II kit in the presence of [α-32P]-dATP. (Feinberg and Vogelstein, 1983). Autoradiographies were produced by exposing the washed membranes to KODAK X-OMAT films.

Table 2.1. List of used strains in this study

Strain	Description	Reference
D141	wildtype, clinical isolate	Staib *et al*., 1980
AfS01	*ΔcpcA*; *phleo*^{R}	Krappmann *et al*., 2004
AfC07	*ΔcdaA*; *hygr*^{R}	this study
AfC08	*ΔcpcB*; *phleo*^{R}	this study
AfC09	*ΔcdaA*; *hygr*^{R}; *ΔcpcB*; *phleo*^{R}	this study
AfC10	*cdaA::loxP*	this study
AfC11	*cdaB::loxP*	this study
AfC12	*ΔcdaA*; *hygr*^{R}*; cdaB::loxP*	this study

Table 2.2. List of used plasmids in this study

Plasmid	Description	Reference
pBluescript®II KS$^+$	cloning vector (*amp*^{R}; MCS)	Stratagene (La Jolla, CA, USA)
pCR®-BluntII-TOPO®	cloning vector (*kan*^{R})	Invitrogen Gmbh (Karlsruhe, D)
pCS5	*cdaA* deletion cassette (*cdaA::loxP-hph/tk*)	this study
pCS8	*cdaB* deletion cassette (*cdaB::loxP-ble/tk*)	this study
pME2891	*loxP-pgpdA::ble/HSV1tk::trpCt-loxP*	Krappmann *et al.*, 2005
pME2892	Cre expression module in pPTRII	Krappmann *et al.*, 2005
pME3002	*loxP-pgpdA::hph/HSV1tk::trpCt-loxP*	Krappmann *et al.*, 2005

Table 2.3. Primers used in this study

Name	Size	Sequence
CS13	19-mer	5’-TGCATCCTGCGCTTCGATG-3’
CS14	19-mer	5’-CAGAATACGCCACGCGAAG-3’
CS17	20-mer	5’-TTCATCCTCTTCCCCACTGG-3’
CS18	23-mer	5’-ACTACGGAAGAAGCGGTCCA-3’
CS21	20-mer	5’-ATACGACCAAATCGATGTCC-3’
CS22	20-mer	5’-AAGTTCAGACTGATCACGTC-3’
CS25	20-mer	5’-AAG GTTCCTTTCGTCTGTGG-3’
CS26	20-mer	5’-GGTCCTTGTAGTGAGTCACG-3’
CS45	20-mer	5’-AGCTGCTTTGTCACTTCCAG-3’
CS46	35-mer	5’-TAGGCCTGAGTGGCCGAAGGAGGATTCCTTGGTGG-3’
CS47	38-mer	5’-TAGGCCATCTAGGCCTACGTCACACGCCAATCCAAATC-3’
CS48	19-mer	5’-CGGAAACGGAGCGAATTAG-3’
CS49	18-mer	5’-CGTGCCTGAGGATCCTCC-3’
CS50	35-mer	5’-TAGGCCTGAGTGGCCAGGGGAAACCTAAGGTAGAG-3’
CS51	34-mer	5’-TAGGCCATCTAGGCCCGAGGAAATCATTCAAAGAC-3’
CS52	20-mer	5’-GGAAGCAAGACAATCTCTGA-3’

CS76	19-mer	5’-GAGCTACGGGATACGATTG-3’

Table 2.3 continued.

Name	**Size**	**Sequence**
CS77	19-mer	5’-ACAAGTCCGTTGAGTTGCG-3’
CS31	20-mer	5’-ATCCATCGTCGCATCAACCC-3’
CS75	18-mer	5’-TGGCGGCCGTTCAGATTG-3’
VG20	18-mer	5’-CTGTTCACGCTGCTGAGC-3’
Sv27	20-mer	5’-TCAATTCACTCAAAAACAGC-3’
Sv48	23-mer	5’-TCTGGAGGATACAATCACTTTCC-3’
Sv49	23-mer	5’-AATCCCATTGAGTGCCTTTCAGC-3’
Sv64	23-mer	5’-TAATCTATTCAAAAGATCTGAGG-3’
Sv65	21-mer	5’-TCTACACACAGATCTAGTTGG-3’

2.4 Results

In the yeast *S. cerevisiae* it was shown that the general control of amino acid biosynthesis with its transcriptional regulator Gcn4p regulates a diverse set of genes and cellular pathways with a certain bias to those involved in amino acid biosynthesis (Delforge *et al.*, 1975; Hinnebusch, 2005; Schurch *et al.*, 1974; Wolfner *et al.*, 1975). Earlier works on transcriptional profiling in dependency of Gcn4p revealed that more than 600 genes are regulated directly or indirectly by this transcriptional activator (Natarajan *et al.*, 2001). The homologous systems to the general control can also be identified in other fungi like *N. crassa* and *A. nidulans*, where it is called Cross-Pathway Control (CPC) (Carsiotis *et al.*, 1974; Davis, 2000; Piotrowska, 1980; Sachs, 1996). In *A. fumigatus*, this system was investigated under the aspect for its role in virulence; thus, previous works showed that the transcriptional regulator CpcA, the orthologue to Gcn4p, is required for full pathogenicity of *A. fumigatus* (Krappmann *et al.*, 2004). Therefore, it was of interest to get an overview of the CpcA-dependent transcriptome with the focus set on stress-related genes. In this study a comparison between a *wild-type* isolate of *A. fumigatus* and its *ΔcpcA* derivative under conditions of histidine starvation was done.

2.4.1 Transcriptional profiling of histidine-starved *A. fumigatus* reveals the CpcA-dependent transcriptome

To analyse the CpcA-dependent transcriptome the *wild-type* clinical isolate D141 and the *ΔcpcA* mutant AfS01 were propagated in liquid minimal medium and shifted to conditions of histidine starvation for four hours induced by the antimetabolite 3AT. Competitive hybridisations on whole genome microarrays were carried out (see Material and Methods) to compare the transcriptional profiles of each strain in the absence or presence of amino acid starvation. Analysis of both transcriptome data sets revealed 1563 genes to be regulated to a certain extent (see supplemental table 1). For further analyses, only genes above a threshold value of 1.5 fold regulation were considered. Accordingly, 1048 entries from the initial gene list showed at least an expression level of 1.5 fold or higher (suppl. table 2). In the *wild-type* 842 genes were found, from which 494 were induced and 348 were repressed in the presence of 3AT; in the *ΔcpcA* strain 493 genes were regulated, from which 195 were up-regulated and 298 down-regulated. Only 290 genes showed in both strains the same regulation. 555 genes are higher expressed in the *wild-type* in comparison to the *ΔcpcA* mutant and 206 are only present in the *ΔcpcA* mutant data set (suppl. tables 3 and 4).

2.4.2 Estimation of the CpcA-dependent transcriptome

In order to identify direct and indirect targets of CpcA, all genes with a regulation level of 1.5 fold or higher (1048) were screened for being regulated in the *wild-type* under starvation condition but not in the *ΔcpcA* strain. 523 gene loci could be assigned to this analysis, resulting in the suggestion that this group contains genes that are regulated by the CPC effector CpcA (suppl. table 5). For validation purposes, the transcriptional steady-state level of some genes induced under starvation was checked in Northern hybridisations. As internal standard a probe binding to the CpcA-independent *aroC* transcript was used. All of the analysed genes were highly expressed in the *wild-type* under starvation but not under normal conditions. Altered expression levels of these genes in the *ΔcpcA* strain were neither seen under starvation nor under non-starvation conditions (fig. 2.1).

The set of 523 genes was classified in functional categories. The majority (377 genes) showed an up regulation in the presence of amino acid starvation (table 2.4 and suppl. table 6), whereas only 146 were repressed under these conditions (suppl. table 7). 49 of the 377 up-regulated genes are involved in amino acid biosynthesis to form the largest functional group of the data set. 48 CpcA-regulated genes encoding putative transporters or permeases could be identified. Also a high number (25) of transcriptional and translational factors were positively regulated by CpcA, corroborating the assumption that CpcA is a master regulator of the fungus. Additionally, genes involved in carbon, nitrogen, and sulfur metabolism could be assigned; in total, 19 genes encoding proteins for these metabolic pathways were detected in the data set. Moreover, factors involved in vitamin and cofactor biosynthesis, fatty acid and purin/pyrimidin metabolism, and secondary metabolism were de-repressed in a CpcA-dependent manner under amino acid starvation conditions as well as genes encoding enzymes related to protein degradation and modification. Furthermore, many genes required for other functionalities were identified, and additional hypothetical genes or genes with unknown function could be found in the data set.

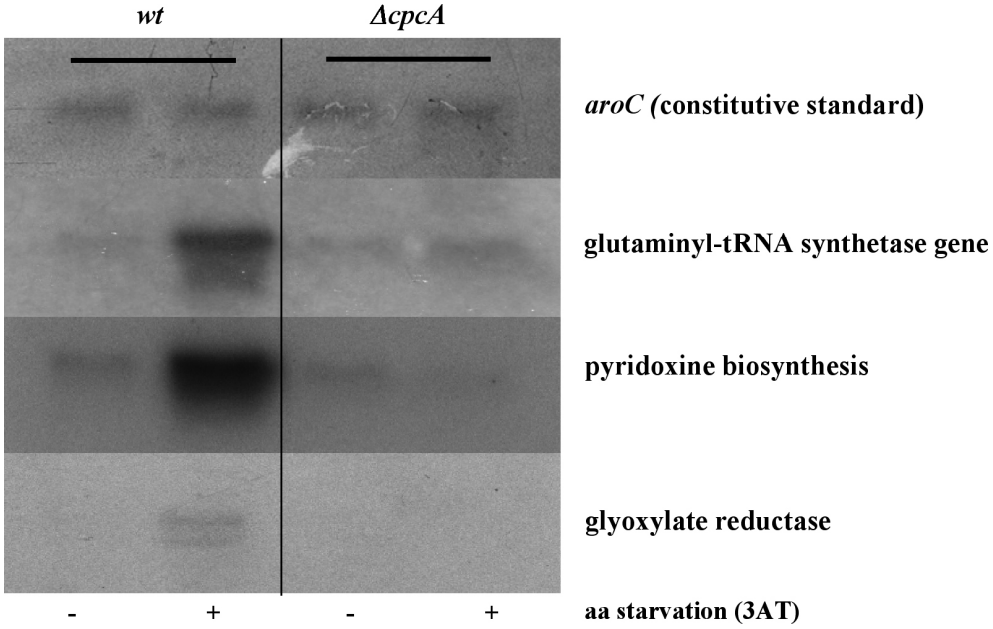

Figure 2.1. Validation of the transcriptome profiling data set
Northern hybridisation patterns of several genes that showed an up-regulation in the transcriptome profile using RNA samples from *wild-type* (*wt*) and the *ΔcpcA* deletion strain in the presence or absence of amino acid starvation conditions. In accordance with the microarray hybridisation data, the selected genes display increased transcription under starvation in the *wild-type* but not in the deletant.

In further analyses, the focus was set on putative CpcA targets that are likely to be involved in any kind of stress response that would support survival of *A. fumigatus* under conditions of environmental stress. In the profiling data, twelve targets could be identified fulfilling this condition (table 2.5).

Table 2.4. Numbers of CpcA-induced genes assigned to functional categories.

Category	Number	% of all 377 induced genes
hypothetical proteins	76	20.2%
aa biosynthesis	49	13.0%
transporters/permeases	48	12.7%
other genes	39	10.3%
transcription-/translation factors	25	6.6%
genes with unknown function	25	6.6%
acetyl-/methyltransferases	14	3.7%
carbon meatbolism	13	3.4%
stress response factors	12	3.2%
purine/pyrimidine-modification/DNA/RNA	10	2.7%
protein degradation	9	2.4%
oxidoreductases involved in metabolism	8	2.1%
tRNA modification/synthesis	8	2.1%

Table 2.4 continued.

Category	Number	% of all 377 induced genes
replication and repair systems DNA/RNA	7	1.9%
coenzyme sythesis	7	1.9%
nitrogen and sulfur metabolism	6	1.6%
secondary metabolism	4	1.1%
heavy metal binding (Cu, Fe, Zn)	4	1.1%
cell growth	3	8.0%
ribosomal proteins	3	8.0%
respiration	3	8.0%
fatty acid metabolism	3	8.0%
vitamin biosythesis	3	8.0%
cell wall	1	0.3%

Table 2.5. CpcA-dependent *A. fumigatus* genes putatively involved in stress response.

Locus tag	Gene
Afu7g05750	Afu7g05750 AAA family ATPase, putative
Afu3g10770	Afu3g10770 RTA1 domain protein, putative
Afu5g07960	Afu5g07960 C2H2 finger and ankyrin domain protein, putative
Afu7g06570	Afu7g06570 zinc/cadmium resistance protein
Afu1g16560	Afu1g16560 MIF domain protein
Afu1g05040	Afu1g05040 protein mitochondrial targeting protein (Mas1), putative
Afu7g06680	Afu7g06680 AAA family ATPase, putative
Afu2g00590	Afu2g00590 glutathione-S-transferase, putative
Afu6g08460	Afu6g08460 cytochrome P450 alkane hydroxylase, putative
Afu3g10220	Afu3g10220 acid phosphatase, putative
Afu5g13000	Afu5g13000 CRAL/TRIO domain protein
Afu2g11180	Afu2g11180 developmental regulator FlbA

In the data set CpcA repressed the expression of 146 genes in the presence of 3AT (table 2.6); 17 of these genes encode transporters or are involved in transport mechanisms. In addition, eleven genes related to carbon metabolism, which was nearly the same number of genes that were up-regulated under starvation were found. In contrast to that, no gene involved in amino acid biosynthesis was found to be repressed and only four transcriptional factors showed repression in a CpcA-dependent manner. Also the identification of annotated genes in the repression data set having a function in nitrogen or sulfur metabolism failed. The comparison of genes involved in protein metabolism and degradation revealed to no obvious difference between the *wild-type* and the deletion strain, since in both data sets targets of these functions

were found. Interestingly, genes classified to be involved in stress-response were also down-regulated in dependency of CpcA. However, these were mainly proteins assigned to chaperone function in order to react on stress induced by heat, whereas in the other data set a strong diversity predominated.

Table 2.6. Numbers of CpcA-dependent repressed genes within different functional categories.

Category	**Number**	**% of all 146 repressed genes**
hypothetical proteins	45	30.8 %
transporters/permeases	17	11.6%
other genes	14	9.6%
genes with unknown function	13	8.9%
stress/heat shock	12	8.2%
carbon meatbolism	11	7.5%
protein degradation	6	4.1%
transcriptionsfactors	4	2.7%
DNA mod./RNA mod./	4	2.7%
ribosomal proteins	3	2.1%
oxidoreductases involved in metabolism	3	2.1%
cell wall	3	2.1%
fatty acid metabolism and ureat cycle	3	2.1%
Acetyl-/Methyltransferasen	3	2.1%
respiration	2	1.4%
heavy metal binding (Cu, Fe, Zn)	2	1.4%
nitrogen and sulfur metabolism	1	0.7%
pH regulation	1	0.7%

2.4.3 The CpcA-directed transcriptome of *A. fumigatus* in comparison to the transcriptional profile elicited by voriconazole treatment

Amino acid starvation represents a stress condition to *A. fumigatus* that triggers a cellular response by de-repression of CpcA expression to result in regulation of various kinds of genes. This wide domain response enables the fungus to react on the encountered stress signal. In order to assess some overlap of the CpcA-directed transcriptome and the one elicited by the antifungal drug voriconazole, the data were compared to the transcriptional profile, in which *A. fumigatus* was incubated for 4 h in the presence of this antifungal drug (da Silva Ferreira *et al.*, 2006). Voriconazole belongs to the azole family of antifungal drugs, which target the fungal ergosterol biosynthesis (Diaz-Guerra *et al.*, 2003). Unknown and

hypothetical genes from the previous data set were not taken in consideration for the comparison; among the remaining genes that were induced in the CpcA-directed transcriptome, 46 showed also an up-regulation of 1.5 fold or higher in the presence of voriconazole whereas 37 were repressed. In the presence of 3AT, the transcriptional level of 48 amino acid biosynthesis proteins was increased in the *wild-type*, with only three of them being also induced by voriconazole treatment. However, the remaining 45 amino acid biosynthesis genes were repressed or displayed no induction of 1.5 fold or higher by voriconazole. The second largest group in the profile of induced genes were putative transporters and permeases. Seven of them were down-regulated, and only five displayed an induction of expression during incubation with voriconazole. Interestingly, five of the twelve putative CpcA-regulated stress genes were induced in the voriconazole profile as well, while seven showed a diminished expression level below the threshold of 1.5 fold regulation or they were not detectable in the voriconazole transcriptome profile (table 2.7). A further interesting fact was the pronounced up-regulation of the *cpcA* gene itself during voriconazole treatment, leading to the suggestion that this drug acts on *cpcA* expression on the level of transcription. However, expression of the *argB*-encoded ornithine carbamoylase activity was not detectable in the presence of the antifungal drug, which indicates that voriconazole increases the transcription of the CPC regulator but does not trigger the CPC response itself.

Table 2.7. Stress genes induced under amino acid starvation and voriconazole-induced stress

Locus tag	**Gene**	**3AT***	**Voriconazole***
Afu7g05750	AAA family ATPase, putative	5.40	2.69
Afu3g10770	RTA1 domain protein, putative	3.98	3.84
Afu5g07960	C2H2 finger and ankyrin domain protein, putative	3.49	1.76
Afu7g06570	zinc/cadmium resistance protein	5.00	4.58
Afu7g06680	AAA family ATPase, putative	3.90	49.03

*induction factor of the gene ater treatment with 3AT/voriconazole

2.4.4 CpcA-dependent stress genes

A dozen genes were identified that are likely to be involved in the fungal stress response (see table 2.5) among them a putative glutathione-S-transferase. In *A. fumigatus* three functional glutathione transferases were characterised by Burns *et al*. (2005) to be involved in detoxification of reactive oxidative species (ROS) like H_2O_2 and of xenobiotics. This detoxification function is similar to the function of cytochrome P450 alkane hydroxylase, another putative stress related gene found in our transcriptional profile. Additional functional stress response genes were up-regulated in our data set, for example a protein containing an

RTA1 domain. It is known from yeast that specific proteins containing such an RTA1 domain enable the organism to react on triazole-containing fungal drugs like itraconazole, resulting in increased resistance. From the pathogenic yeast *C. glabrata* it is known that *RTA1* is a target of Pdr1, a transcriptional regulator for pleiotropic drug resistance (Vermitsky *et al.*, 2006). In addition to these stress response proteins, a zinc/cadmium resistance protein encoded by the gene with the locus tag Afu7g06570 was identified. Previous studies had indicated that although these kinds of gene products are not unconditional essential for the organism they are often required for normal growth under stress conditions. Deletion of these genes may lead to accumulation of zinc, which results in a higher sensitivity towards other growth-inhibiting drugs. This was shown, for instance, for a zinc transporter in *S. cerevisiae* encoded by the *YKE4* gene, whose deletion results in zinc accumulation accompanied by a higher sensitivity to Calcofluor White in medium that also contains increased concentrations of zinc (Kumanovics *et al.*, 2006). A further interesting gene found in the data encodes a regulator involved in development (*flbA*). This gene, which belongs to the family of regulators of G-protein signalling (RGS) was characterised in *A. nidulans* as a negative regulator for vegetative growth and to be involved in signalling in order to react on changing environmental conditions (Lee and Adams, 1994; Yu *et al.*, 1996). The other RGSs *rgsA*, *rgsB* and *rgsC*, which are also known from *A. nidulans* (Han *et al.*, 2004) were however not detected in the data set.

Among the twelve different stress genes, two genes encoding ATPases belonging to the AAA-ATPases family are present. Members of the AAA-ATPase family are associated to various cellular activities like chaperone activity, proteolysis and movement of microtubule motors in eukaryotes. Most of these proteins undergo a conformation change and/or bind ATP for hydrolysation, resulting in physical works; however, the exact mechanism of conformational changing is still not completely clarified. In general, proteins of the AAA-ATPases family consists of six different domains, and they have the ability to form oligomers, in general hexamers. Most eukaryotic genomes encode 50 to 80 members of this protein family (Hanson and Whiteheart, 2005). Accordingly, BLAST analysis using the typical conserved AAA-motif, as input query was carried out against the *A. fumigatus* genome to retrieve eleven annotated gene loci encoding putative members of this family (table 2.8). Interestingly, two of these ATPases were induced in the transcriptional profiling studies, accordingly they were designated *cdaA* (Afu7g06680) and *cdaB* (Afu7g05750) for *C*pcA-*d*ependent AAA+ *A*TPases. For further investigations and characterisation the up-regulation of these both genes upon 3AT treatment in a CpcA-dependent manner had to be validated.

Table 2.8. AAA ATPases of *A. fumigatus*

Afu1g02060	Afu2g12920	Afu4g11180	**Afu7g06680**
Afu1g02410	Afu4g04800	Afu6g12560	Afu8g04270
Afu1g09210	Afu4g10730	**Afu7g05750**	

The genes induced under amino acid starvation conditions and by voriconazole-treatment are written in bold.

2.4.5 *cdaA* and *cdaB* appear to be regulated by the transcriptional activator CpcA

To check the induction of both AAA-ATPases in dependency of CpcA under starvation conditions, Northern hybridisation experiments were carried out. Therefore, the wild-type isolated D141 and in parallel the *cpcA* deletion strain AfS01 were cultivated in liquid culture in the absence or presence of 3AT. As internal standard, transcript levels of the CpcA-independent gene *aroC* were used (fig. 2.2A). The wild-type displayed increased signal intensities for both AAA-ATPases in comparison to the sample without 3AT, and no signal could be detected for the *ΔcpcA* strain in the presence of this antimetabolite. To exclude that this was a phenomenon depending specifically on 3AT, further controls were performed, but in contrast to the previous ones MSX (L-Methione sulfoximine) was used as inducer of the CPC. This drug inhibits the glutamine synthetase to result in glutamine starvation. With respect to CpcA-dependent regulation of transcription, no difference to the 3AT-treated samples were observed for *cdaA*. The *cdaB* gene, however, showed under this condition not only a prominent signal in the wild-type but also in the mutant (fig. 2.2B). These data indicate that expression of both genes is affected by amino acid starvation conditions in a positive manner; however, only for the *cdaA*-encoded AAA+ ATPase a CpcA-dependency gene regulation could be validated.

For both gene products the AAA motif was found at the C-terminus, and each encoding gene consists of two exons separated by one intron. In addition the promoter regions of these genes within 1000 basepairs (bp) were analysed for typical *cpcA* binding sites (5'-TGACTCA-3'; 5'-TGACTgA-3'; 5'-TGACTgA-3'; 5'-TGAgTgA-3'; Tian *et al*., 2007) to support whether they are putative targets of CpcA. For both genes, one conserved binding site could be detected. However, only in the *cdaA* promoter region a recognition site of the canonical 5'-TGACTCA-3' sequence was identified, whereas in the *cdaB* upstream region a site of the less typical CpcA binding region 5'-TGAgTgA-3' was found (fig. 2.3). This result hints towards a direct regulation of the *cdaA* gene by the transcriptional activator CpcA and a less stringent one for the *cdaB* gene. Furthermore, we were interested in the cellular function of both ATPases in *A. fumigatus*; thus, both genes were deleted in additional experiments.

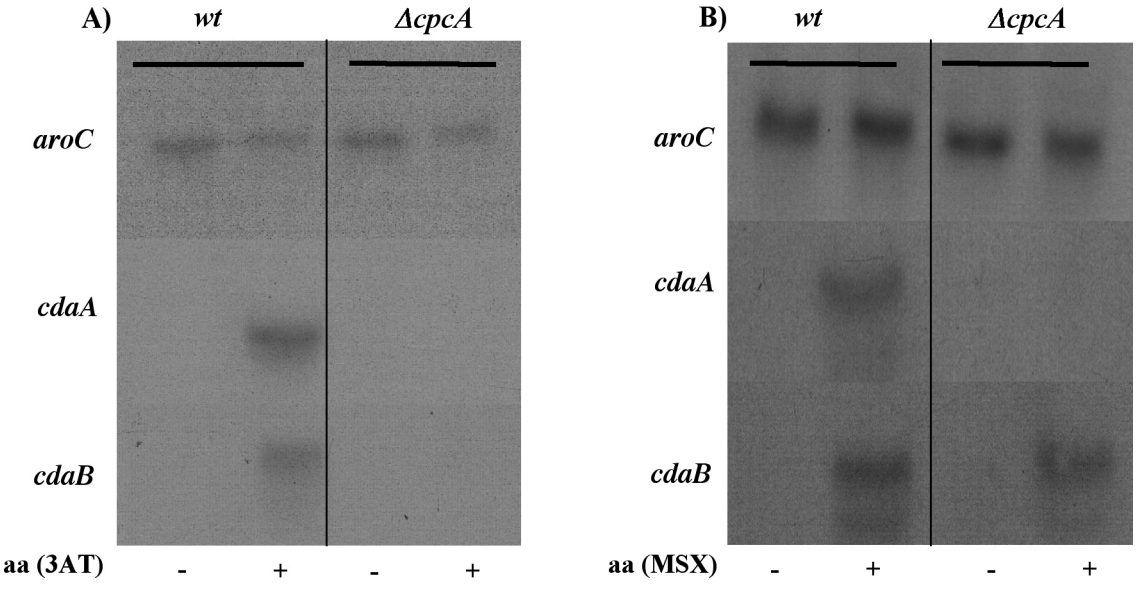

Figure 2.2. Northern hybridisations of the two AAA-ATPases *cdaA* and *cdaB* under amino acid starvation
A) Induction of both *cda* genes under starvation induced by 3AT in the *wild-type* strain D141. No signal was detected in *cpcA* deletion strain with and without starvation as well as in the *wild-type* under non-starvation. B) Transcription of *cdaA* and *cdaB* was also increased under amino acid starvation induced by MSX but not under sated conditions; in contrast to *cdaA*, *cdaB* appeared also up-regulated in the *ΔcpcA* mutant starved for the amino acid glutamine. Accordingly, only for the *cdaA*-encoded gene a CpcA-dependent regulation of transcription was evident. Steady-state levels of the *aroC* gene were used as internal standard in the Northern hybridisations.

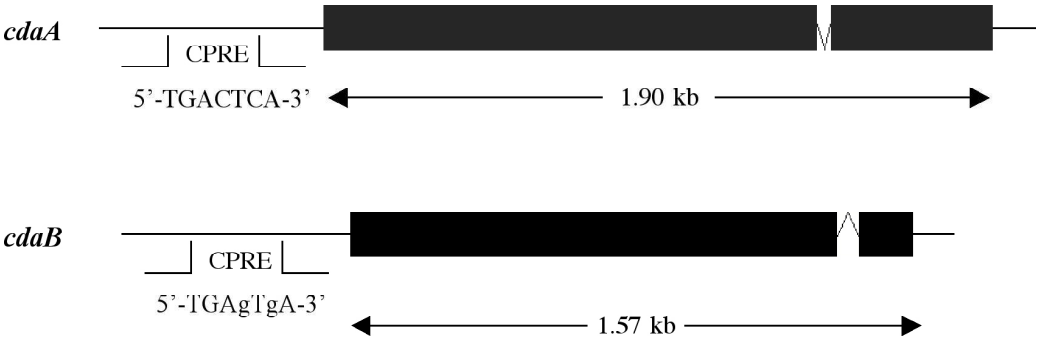

Figure 2.3. *cdaA* and *cdaB* with their putative CpcA binding sites
The figure displays an overview of the two genes *cdaA* and *cdaB* with their promoter region. *cdaA* has a size of 1.90 kb and contains within 1kb of its promoter region the cantoning sequence 5'-TGACTCA-3' as putative CpcA binding region. *cdaB* is with 1.57 kb a bit smaller and contains not the high conserved binding region in its promoter. The 5'-TGAgTgA-3' sequence is also a putative recognition site for the *cpcA* gene product but has a less affinity to this protein than 5'-TGACTCA-3'.

2.4.6 Deletion of *cdaA* and *cdaB* results in increased sensitivity towards Calcofluor White

To generate a *ΔcdaA* deletion strain, a recyclable knock out cassette described by Krappmann *et al*. (2005) conferring hygromycin resistance was used. To delete the *cdaB* gene, a comparable construct was used containing a phleomycin resistance (fig. 2.4). Both deletion cassettes were homologously integrated in the D141 wild-type isolate. Moreover, a double knock-out of both genes was made in the D141 background (see Materials and Methods).

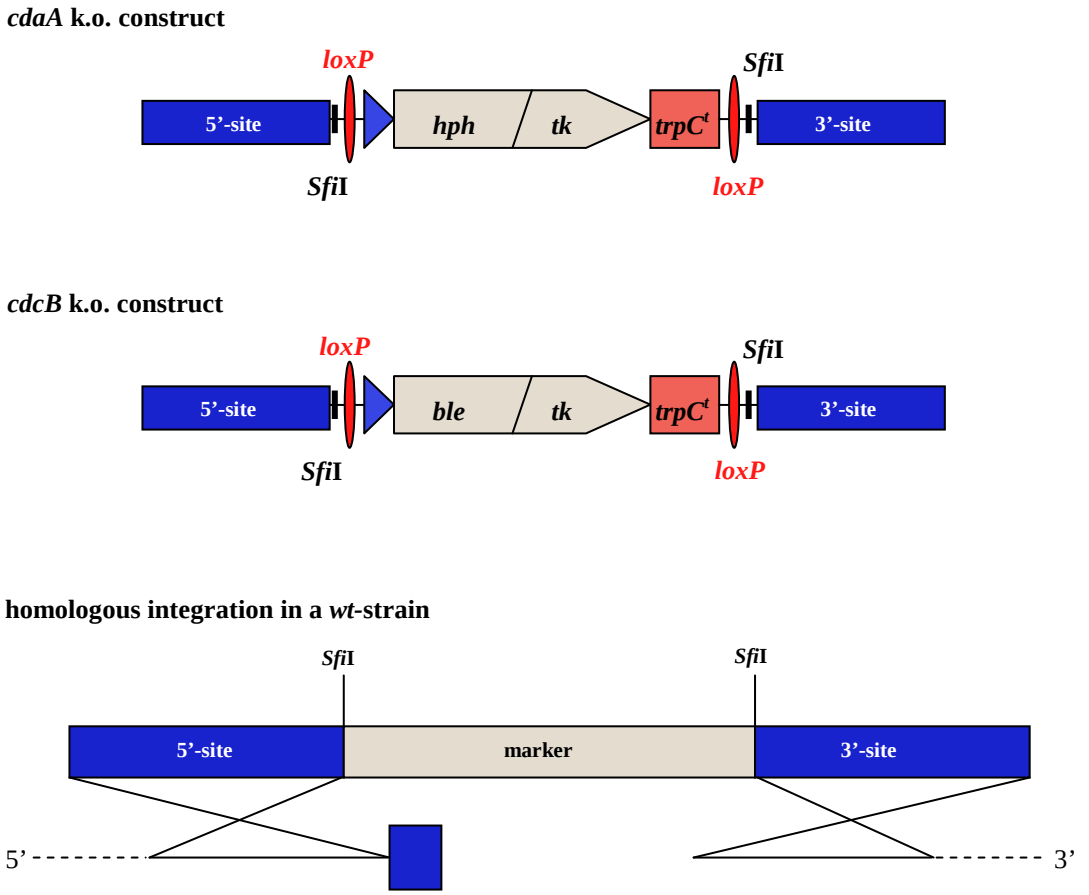

Figure 2.4. Construction of *cdaA*, *cdaB* and the *cdaA/B* deletion strains
For constructing the deletion cassettes we used a hygromycin resistance for the *cdaA* and a phleomycin resistance for the *cdaB* gene. *Via Sfi*I restriction sites the 5' and the 3' flanking regions of these genes were connected to the marker cassettes. *Via* homologous integration the genes were deleted. For counter selection on FUDR the construct contained a thymidin kinase and to make the whole system recyclable there were two *loxP* sites at the flanking regions of the deletion construct. To create the double deletion (*cdaA/B*) the *cdaA* knock-out strain was used.

For the identification of correct deletants, candidates from the transformants' pool were tested by Southern hybridisation. To check for the *cdaA* deletion, a probe binding to the 5' region of the gene was used. Genomic DNA isolated from clonal transformants was digested with the

enzymes *Nco*I and *Pvu*II, for comparison *wild-type* DNA was used. One transformant displaying the expected signals at 4.4 kb (*NcoI*) and 5.7 kb (*Pvu*II) was used for further experiments (fig. 2.5A). To check for deletion of the other *cda* gene, a probe binding to the 5' region of *cdaB* was constructed and used in the Southern hybridisations. Here, the enzymes *Sph*I and *Ssp*I were used for restriction. Signals indicating the correct deletion situation should be at 2.5 kb (*Sph*I) and 6.1 kb (*Ssp*I) (fig. 2.5B). For the double deletion, the *ΔcdaA* isolate was used and transformed with the *ΔcdaB* replacement cassette. For final validation Southern hybridisation was made with the same probes and enzymes as for the two single knock-outs, and isolates resulting in the correct bands were used for further investigation (fig. 2.5C).

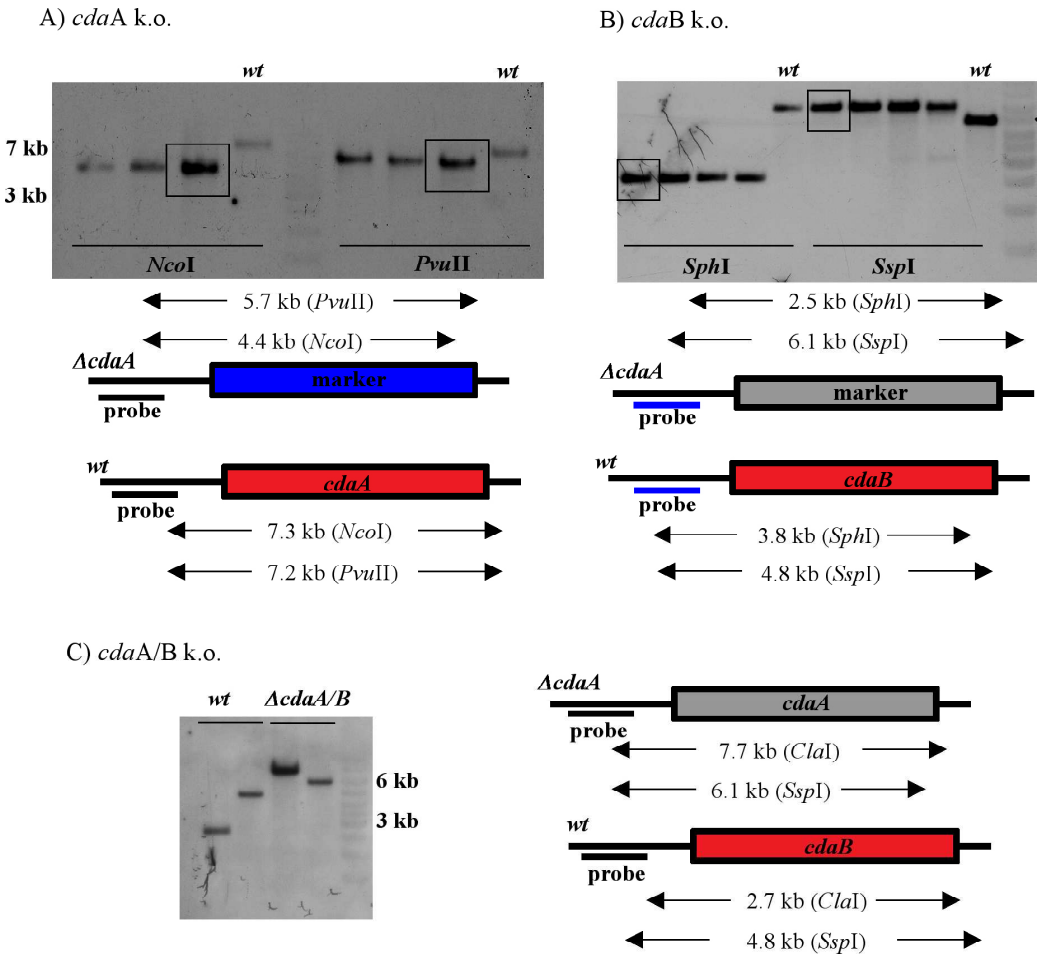

Figure 2.5. Validation of *cda* deletions strains by Southern hybridisation
A) Genomic DNA of putative *cdaA* knock out clone was digested with *Nco*I and *Pvu*II resulting in the expected signals at 7.3 and 7.2 kb by using a probe binding at the 5′ region of *cdaA*. As control the wildtype was used. B) DNA from putative *ΔcdaB* strains were digested with *Sph*I and *Ssp*I resulting in bands with a size of 2.5 and 6.1 kb for right clones. The prepared probe binds at the 5′ region of *cdaB*. C) Double knock out was made in a *cdaA* deletion background. Clones were tested by digestion of genomic DNA with *Cla*I and *Ssp*I. Correct deletions lead to signals at 7.7 and 6.1 kb.

The integrated resistance cassettes in the deletions strains were recycled by transforming a plasmid expressing the Cre recombinase (Krappmann *et al.*, 2005) into the deletion strains. For counterselection purposes, putative clones were plated on FUDR-containing medium (see Materials and Methods), and Southern experiments were executed for validation. To check for recycling of the marker from the *ΔcdaA* deletion strain genomic DNA was digested with *Nco*I, and correct clones should show a signal at 5.2 kb. For recycling *ΔcdaB* in the single and double deletion strains genomic DNA was digested with *Ssp*I to get a signal at 3.3 kb for correct clones. Moreover, isolates carrying the deletions of *cdaA* and *cdaB* without the corresponding resistance cassette for additional experiments were attempted, but only the phleomycin resistance cassette could be recycled (fig. 2.6A-C).

In additional experiments a set of deletants was tested for growth on different kinds of media. As control and for comparison the *wild-type* progenitor as well as the *cpcA* deletion strain were used. On minimal medium no obvious growth phenotype was evident (fig. 2.7A). Under amino acid-limiting conditions using MSX-containing media both *Δcda* deletion strains grew as well as the *wild-type*, whereas the *ΔcpcA* displayed reduced growth (fig. 2.7B). In contrast to this, all knock out strains displayed sensitivity to Calcofluor White (CW), an inducer of cell wall and septa stress: on medium containing 0.1 mg/ml CW, *cda* deletion strains and the *ΔcpcA* mutant show reduced growth in comparison to the wild-type isolate (fig. 2.7C). Interestingly, the *ΔcdaA* mutant carrying the hygromycin resistance cassette displayed a less stringent phenotype in comparison to the *cdaA::loxP* strain. Accordingly, it can be suggested that the presence of this particular resistance cassette contributes to a certain extent to resistance towards cell wall stress induced by Calcofluor White. This phenomenon was not detectable for the phleomycin resistance in the *ΔcdaB* deletion strain or in the *ΔcdaA; cdaB::loxP* mutant. Based on these results the two AAA-ATPases seem to be involved in cell wall stress resistance.

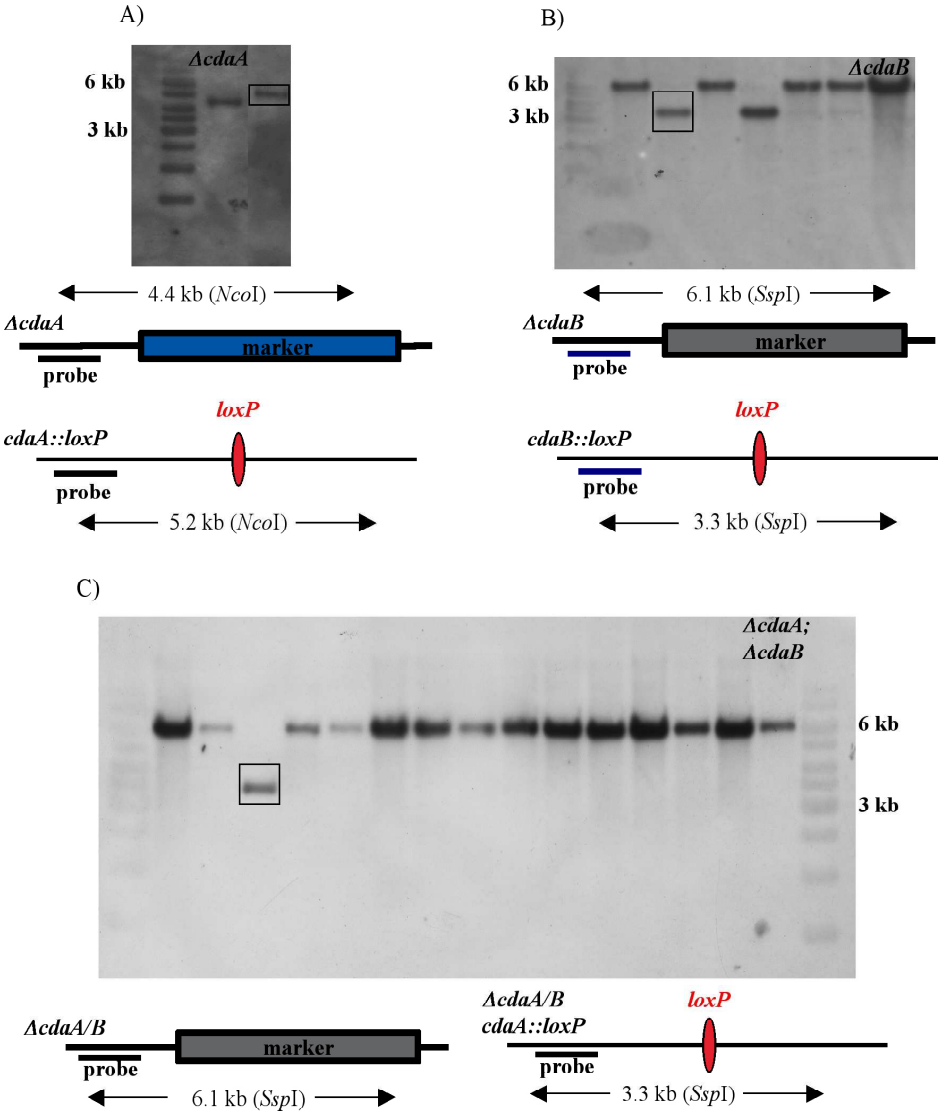

Figure 2.6. Southern hybridisation of the recycled marker systems in *cdaA*, *cdaB* and *cdaA*; *cdaB*
A) The Southern hybridisation displays a *cdaA* deletion clone loosing its resistence cassette after transformation with a plasmid containing a Cre recombinase. Digestion was done with *Nco*I resulting in signals at 5.2 kb for the right clones. As control we used the *cdaA* deletion strain with the resistance cassette. B) Digestion with *Ssp*I of different *ΔcdaB* clones after recycling the resistance cassette. Correct clones should have the signal at 3.3 kb. As control the *ΔcdaB* strain containing the dominant marker was used. C) Southern hybridisation of *cdaA/B* deletion strains with and without the *cdaB* marker cassette. Digestion with *Ssp*I leading to a 3.3 kb signal for the clone without marker.

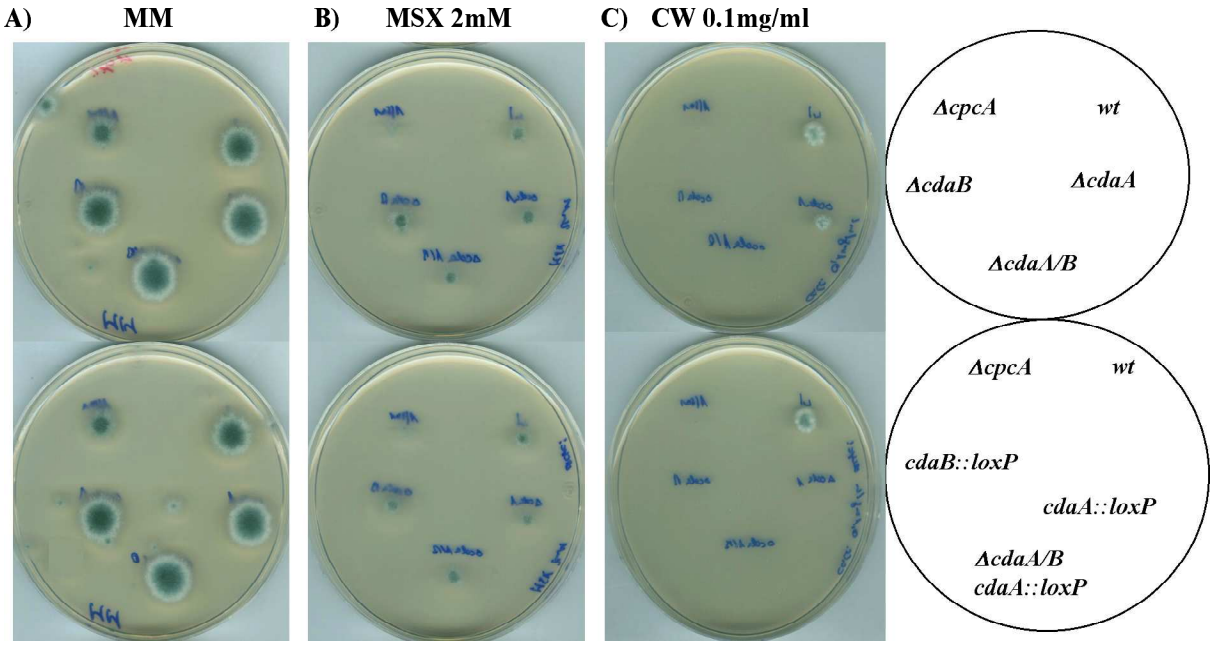

Figure 2.7. Growth test of *cda* deletion strains on different media
A) *cda* deletions strains with and without marker, the wt and the *ΔcpcA* plated on MM. All strains showed a normal growth on this medium. B) Under amino acid starvation by using MSX a phenotype was only visible for the *ΔcpcA* strain. The Δ*cda* mutants grew as well as the *wild-type*. C) On CW containing medium all mutants showed a growth defect in contrast to the *wildtype*. Strains having still the hygromycine resistance cassette showed an attenuated phenotype.

2.4.7 Calcofluor White has no inductive effect on the Cross-Pathway Control

According to the fact that CpcA regulates two AAA-ATPases under amino acid starvation and that deletions of these genes lead to an increased sensitivity to Calcofluor White, the question of CW as CPC-inducing agent was followed. Therefore, the wild-type and the *ΔcpcA* deletion strain were inoculated in minimal medium, after 18 h. Afterwards the mycelia were shifted to medium with 0,002 mg/ml Calcofluor White and incubated for additional 4 h. From these cultures samples for RNA extraction were taken. Northern experiments revealed that transcription of the *cpcA* gene is slightly induced in the *wild-type* under stress conditions compared to the untreated sample (fig. 2.8A). RNA samples extracted from cultures induced with a higher concentration of CW (0,004 mg/ml) were hybridised with a probe binding to the ornithine carbamoyle transferase-encoding gene *argB*, which is regulated by CpcA and therefore an established indicator for the CPC status. Up-regulation of *argB* gene transcription was not evident (fig. 2.8B), indicating that the Cross-Pathway Control is not induced by cell wall stress conditions.

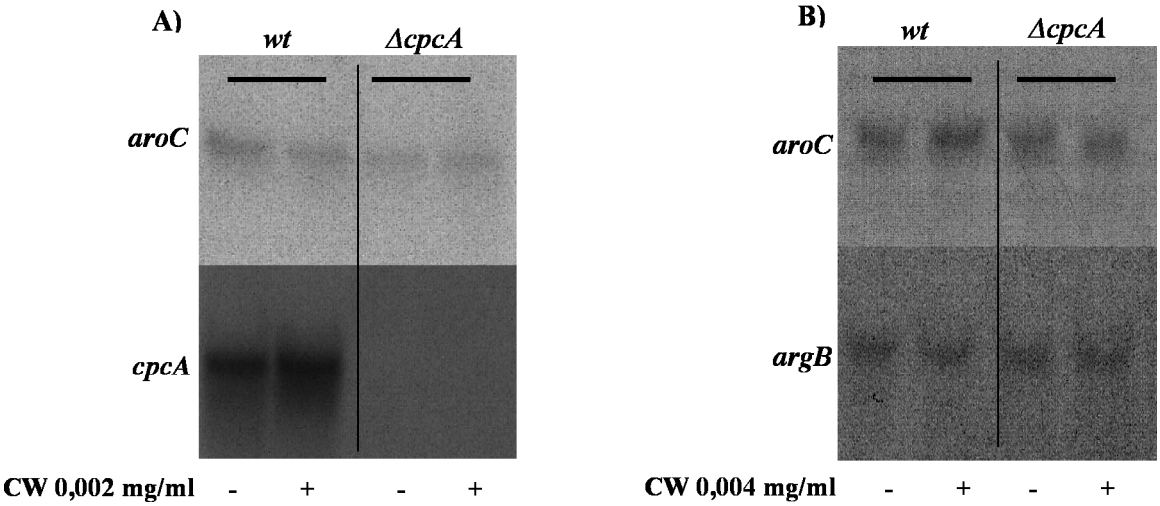

Figure 2.8. Northern hybridisations of the *cpcA* and the *argB* under Calcofluor White conditions
A) Under cell wall stress by using 0.002mg/ml Calcofluor White (CW) is the transcriptional level of *cpcA* slightly induced in comparison to non-stress conditions. No signal was visible in the *ΔcpcA* strain with and without stress. B) Induction with Calcofluor White (CW) (0.004mg/ml does not induce the *argB* gene, an indicator for the induction of the CPC, in the wild-type. No induction was also visible in the *cpcA* deletion strain. In both Northern experiments the CpcA independent *aroC* gene was used for internal standard.

2.5 Discussion

The Cross-Pathway Control system of filamentous fungi is an adaptive system that enables the organism to react on different kinds of environmental stress. Interestingly, this system is required for full virulence of *A. fumigatus* (Krappmann *et al.*, 2004). Therefore, the main focus in these studies was to analyse the CPC transcriptome executed by CpcA in the absence and presence of amino acid starvation conditions. These two profiles were compared with a data set generated from the *ΔcpcA* deletion strain under starvation and non-starvation conditions with respect to amino acids. Resulting from both transcriptional experiments 523 genes were assigned that appeared to be regulated by the transcriptional regulator CpcA. The majority of them (377) were up-regulated in their transcription and only 146 displayed repression of expression. As expected, many amino acid biosynthesis genes were strongly induced in this study. Especially genes involved in the arginine biosynthesis pathway like the *argJ* and the ornithine carbamoylase-encoding *argB* gene were highly induced under amino acid starvation. These results are comparable to the data of *N. crassa* and *S. cerevisiae*, in which also a high proportion of genes involved in amino acid biosynthetic pathways were de-repressed under amino acid starvation conditions (Natarajan *et al.*, 2001; Tian *et al.*, 2007). These genes demonstrate the direct response of the CPC/GC to the internal stress signal. The ribosomal machinery is required to enable a high translation of the amino acid biosynthesis genes. Therefore it is not surprising that three genes which are required for the ribosoms were also up-regulated in our data. The induction of genes encoding ribosomal components of *S. cerevisisae* was also shown by Natarajan *et al*. (2001). Investigation of the whole data set reveals that a lot of different metabolic pathways are induced under these starvation conditions. From yeast and *A. nidulans* it is known that other starvation conditions such as glucose deprivation or purine limitation also have an effect on the CPC/GC regulation (Rolfes and Hinnebusch, 1993; Yang *et al.*, 2000). Deduced from the strong diversity of metabolic pathways that are under control of the transcriptional activator CpcA it becomes clear that the CPC is a global regulator system in fungi like *A. fumigatus*. We found genes involved in sulfur and carbon metabolism as well as in purine biosynthesis. Also genes of the main catabolic pathways like glycolysis or fatty acid utilisation were detected that show an induction under amino acid starvation. These results and observations leading to the conviction that this stress response system reacts in a non-specific manner thus a lot of genes are activated which are not directly required to engage the stress conditions. Nevertheless, this system enables the fungus to react on many different kinds of stress signals that until now cannot be estimated. The high number of CpcA targets make studies on the CPC a

challenging task. More than 5% of all existing genes in *A. fumigatus* are targets of this regulatory system and its transcriptional regulator. An explanation for this high number of induced genes can be that CpcA has also the ability to activate other transcriptional regulators (25) leading to a signalling cascade of induced genes. Thus the CPC consists not only of direct targets of CpcA but comprises also a considerable proportion of indirectly regulated factors.

As mentioned above many different genes are induced under amino acid starvation. In this study it was possible to find not only genes involved in typical metabolism pathways but also in other cellular functions. For instance, a large group of genes encoding transporters and permeases are also increased in their expression under starvation conditions. Some of them are involved in nutrient uptake from the environment. Permeases for amino acids, purines and uracil as well as an oligopeptide transporter were found. It is known from *C. albicans* and *S. cerevisisae* that oligopeptide transporters are required for taking up small peptides from the environment (Hauser *et al.*, 2001; Reuss and Morschhäuser, 2006). Interestingly, also many transporters that are not involved in nutritional supply were activated under starvation. Most of these transporters belong to the ABC, MSF and MFS multi drug transporter families. Although the function of all these transporters and pumps are not clear, it can be suggested that many of them are involved in a sort of stress or drug response (Andrade *et al.*, 2000a; Andrade *et al.*, 2000b; Del Sorbo et al., 2000; Nakaune *et al.*, 2002; Tobin *et al.*, 1997). Accordingly, the fungus gains the ability to pump toxic molecules coming from the environment out of the cell, by which the organism achieves a higher possibility to survive. Consequently, the CPC is not only a system that enables the fungus to live and grow under limited nutrient conditions but also to react and neutralise toxic molecules.

Furthermore, in the data set genes encoding enzymes with protein degradation function were detected, which may be involved in intracellular turnover of proteins. One of these is an ubiquitin-conjugating enzyme. For this kind of proteins it is known that they are required to tag proteins for subsequent degradation (Muratani and Tansey, 2003). Additionally, some proteins of protease activity were detected, which degrade structures and macromolecules outside the fungus in the near environment to result in freely available resources which then can be assimilated by special transport systems (see above) (Schaal *et al.*, 2007).

The number of CpcA-dependent repressed genes is significant lower than the one of induced ones. This is not surprising since CpcA is a transcriptional activator. Accordingly, the major effect of repression comes from other transcription factors regulated by CpcA. Most of the repressed targets in the data set are not involved in metabolism required to grow under limited

conditions: no amino acid biosynthesis genes and only one gene involved in nitrogen metabolism could be found. Interestingly, three transporters required for the iron uptake system were found to be repressed. This implies that the Cross-Pathway Control represses iron uptake and that the involvement of the transcriptional activator CpcA does not result in positive regulation of the iron transport system, which is essential for virulence of *A. fumigatus* (Schrettl *et al.,* 2004). It also seems to be true for heat tolerance: genes were found to be repressed under amino acid starvation that play a role in a kind of heat stress response. Additional repressed stress factors appeared to a low extent in the transcriptome profiles.

The main focus in this study was set on the detection of positively regulated stress genes, which may be required for virulence of the fungal pathogen. Twelve putative stress genes could be deduced from the data sets. Their possible functions differ from detoxification of reactive oxidative species (ROS) to drug resistance. In addition, two AAA-ATPases with yet unknown function were found. These proteins can affect diverse pathways and mechanisms and are often required in cellular stress response pathways (Hanson and Whiteheart, 2005). By further investigation it was corroborated that both genes are targets of CpcA under amino acid starvation conditions induced by 3AT and that both genes contain a putative CpcA binding site in their promoter region. This leads to the suggestion that they are direct targets of the transcriptional activator. Further on, both genes were deleted and also a double knock-out mutant could be generated with the aim to assign any cellular function to the corresponding gene products. Growth tests of the *cda* deletion strains displayed a reduced growth on Calcofluor White-containing medium, which induces cell wall stress for the fungus. In order to confirm the observed phenotypes, further experiments including reconstituted strains have to be carried out. In contrast to this observation, no phenotype could be detected with respect to amino acid starvation for these deletion strains. Also induction of the CPC system under cell wall stress induced by CW treatment failed. Interestingly, slight induction of the CpcA enconding gene was detected under this kind of stress condition. From these preliminary data it may be deduced that the *cpcA* gene can be induced independently from the Cross-Pathway Control. This is supported by the transcriptional profile of *A. fumigatus* under antifungal drug conditions using voriconazole as published by da Silva Ferreira *et al*. (2006), where *cpcA* expression was also induced but typical genes for amino acid biosynthesis were not regulated.

In summary it can be stated that the CPC is a global regulatory system that affect more than 500 genes in order to give the fungus the ability to react on many different kinds of stress and starvation. Moreover, expression of the transcriptional activator CpcA, an integral part of this

regulatory system can be induced independently from amino acid starvation conditions, which are the prime stress conditions to trigger the Cross-Pathway Control in fungi.

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Chapter 3

Basal Expression of the *Aspergillus fumigatus* Transcriptional Activator CpcA is Sufficient to Support Pulmonary Aspergillosis

3.1 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.

3.2 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 (Brakhage, 2005; Walsh and Groll, 2001). 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 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; Hoffmann *et al.*, 2001; Wanke *et al.*, 1997). In general, binding of accumulated uncharged tRNA molecules, that mirror amino acid starvation, to the GC/CPC sensor kinase Gcn2p 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.

3.3 Materials and Methods

3.3.1 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^-, *ΔlacX74*, *galE*, *galK*, *thi1*, *rpsL*, *ΔphoA*(*Pvu*II)] 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 3.1. 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.

3.3.2 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 BIORAD 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).

3.3.3 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.* (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 (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 *EcoR*V 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 *Fsp*I/*Spe*I

fragment comprising the *cpcC* 5' region was inserted into pGEM5(+) *via* *Eco*RV/*Spe*I, followed by insertion of a 3' *Pst*I fragment (5.1 kb) into the *Mph*1103I site. The resulting plasmid pME3287 was digested with *Nde*I and *Not*I, 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 recombineering 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 *Sal*I/*Xho*I digestion into the *Sal*I site of pME2563, yielding pME3289. From this construct, a 9 kb fragment was released *via* *BssH*II 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.

3.3.4 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 (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).

3.3.5 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 $2x10^5$ 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 (Aufauvre-Brown *et al.*, 1998; Tang *et al.*, 1993). 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.

3.3.6 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.

3.3.7 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).

Table 3.1. Strains, plasmids and oligonucleotides used in this study

Name	**Description or sequence**
Strains	
D141	*Aspergillus fumigatus wild-type* strain (syn. NRRL 6585), clinical isolate
AfS01	strain D141 carrying deletion of *cpcA* coding sequence: $Phleo^r$, MSX^s
AfS26	strain D141 carrying deletion of *cpcC* coding sequence: $Phleo^r$, MSX^s
AfS27	AfS26 with excised marker module: $Phleo^s$, MSX^s
AfS55	reconstituted AfS01 expressing *gfp*-tagged *cpcA* allele: $Phleo^s$, MSX^r
Plasmids	
pBluescript II KS	general cloning plasmid [*bla*, multiple cloning site]

Table 3.1 continued.

Name	**Description or sequence**
pGEM5(+)	general cloning plasmid [*bla*, multiple cloning site]

pPTRII	autonomously replicating Aspergillus plasmid [*ptrA*, *AMA1*, *bla*]
pKOBEG	pSC101 derivative expressing l phage *redgba* operon from pBAD promoter
pMCB17	GFP gene (*pyr-4::*^{p}*alcA::gfp2-5*) in pUC19
pME2563	*cpcA* allele carrying silently mutated *Sal*I site in coding sequence
pME2891	*loxP-phleo*^{r}*/tk* blaster [*loxP-*^{p}*gpdA::ble/HSV1 tk::trpC*^{t}*-loxP*]
pME2892	Cre expression module in pPTRII [*A. nid. niaD::cre*, *ptrA*, *AMA1*]
pME3286	genomic *cpcC* locus as 6.1 kb *Dra*I/*Nae*I fragment in pGEM5(+) *Eco*RV
pME3287	template for construction of *cpcC* deletion cassette by recombinatorial cloning
pME3288	*cpcC::loxP-phleo*^{r}*/tk-loxP* replacement cassette for complete deletion
pME3289	construct for *cpcAD* reconstitution by *gfp::cpcA* allele
Oligonucleotides	
Sv117	5’-AGA CCT ACT GAA TCT GGG TTC AGA CTT TCT ACA CCG CCC ACC GCC CCG CTC AGC TGA
Sv118	5’-ACT TCA ATA CAA ATA GAA CAT AAC ATG GTA CTT CGT CAC TCG TTG ATC CTG CAT AGG
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’

3.4 Results

3.4.1 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 *Aspergillus 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. 3.1A). 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. 3.1B). However, when phagocytosis was monitored in the presence of amino acid starvation, as induced by the histidine analogue 3-amino-1,2,4-triazole (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. 3.1C). 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.

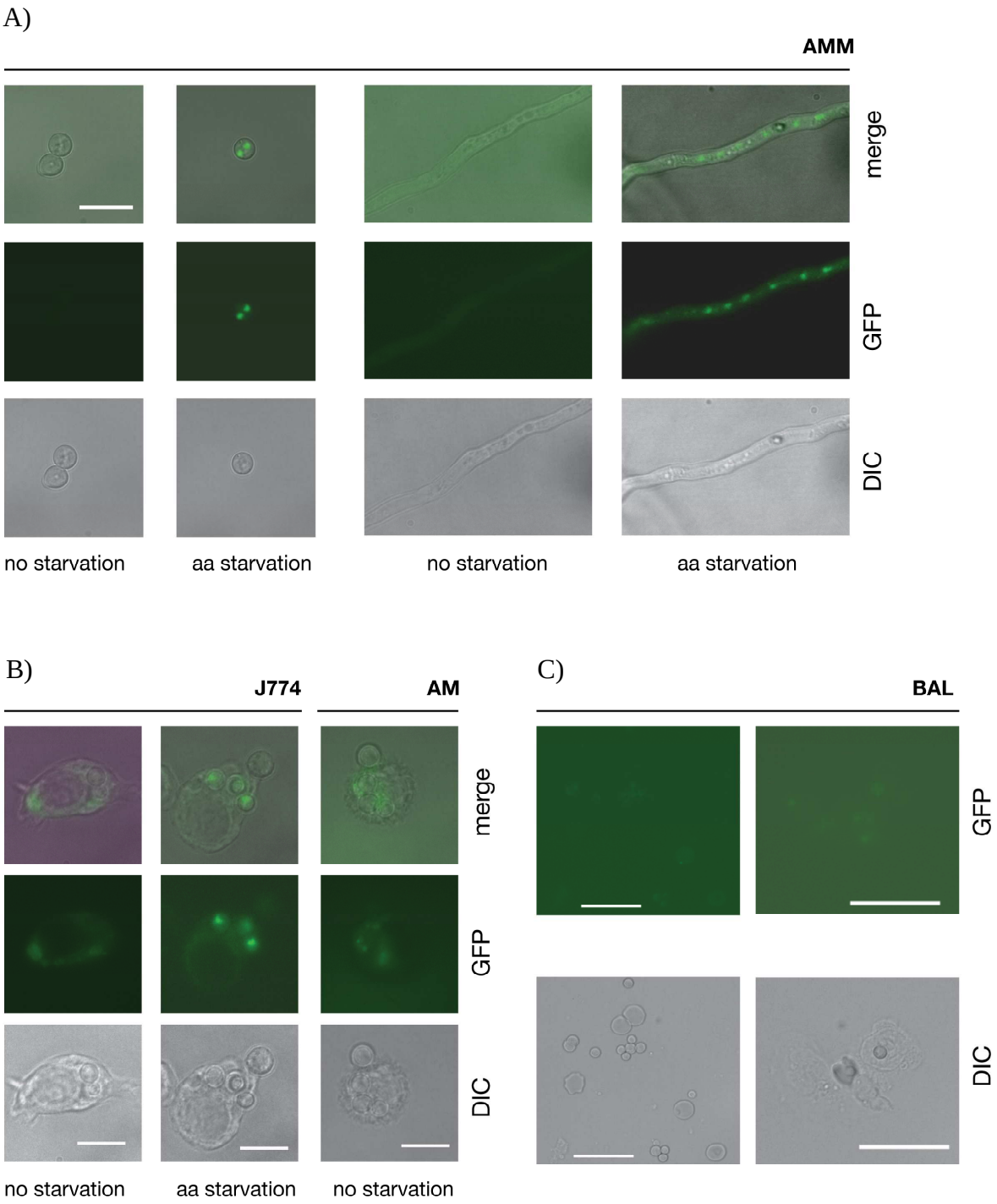


Figure 3.1. The Cross-Pathway Control response in *A. fumigatus* conidia is not de-repressed during confrontation with macrophages or infection of immunocompetent mice
Shown are representative images of the *A. fumigatus* Cross-Pathway Control reporter strain AfS55 [*gfp::cpcA*] propagated in *Aspergillus* 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.

3.4.2 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. 3.2). 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* (Sattlegger *et al.*, 1998; Wanke *et al.*, 1997). 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 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.

Figure 3.2. 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 (Harding *et al.*) and the sensor domain resembling a histidinyl-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.

3.4.3 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. 3.3A). Several descendants were sub-cultured from the pool of primary transformants that could be isolated on corresponding selective

media, and comprehensive Southern analyses confirmed the desired *ΔcpcC* 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 (Krappmannn *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. 3.3B).

A)

B)

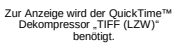


Figure 3.3. Generation of *A. fumigatus cpcCΔ* strains
Deletion strains of the *A. fumigatus* wild-type isolate D141 were generated by gene replacement with a recyclable resistance cassette. A) Outline of the *wild-type cpcC* gene locus in strain D141 and after replacementas present in strain AfS26; 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.

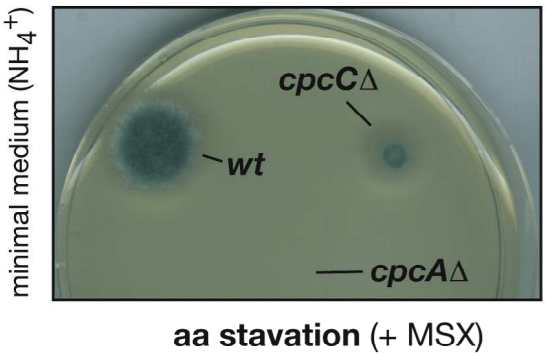
In a first test the growth behaviour of the *ΔcpcC* deletion mutant was evaluated in the presence of amino acid starvation conditions. For this purpose, strains were inoculated on minimal medium containing the drug methionine sulfoximine (MSX), a glutamine synthetase inhibitor. As reference strains the *wild-type* progenitor D141 as well as a strain lacking the CPC transcriptional activator CpcA (AfS01) were also inoculated (fig. 3.4A). 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 *ΔcpcA* 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 [*ΔcpcA*] and high level expression in D141 [*wild-type*] - this observation is in good agreement with the proposed model of CpcA expression (see *Discussion*).

To evaluate the CPC response of the *ΔcpcC* strain in more detail, steady-state levels of reporter transcripts were determined in Northern blot hybridisations (fig. 3.4B). For that purpose, pre-grown mycelia were split and transferred into fresh minimal medium with or without a false feedback inhibitor of histidine biosynthesis (3-amino-1,2,4-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 *ΔcpcC* 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 situation in the ascomycete *Neurospora 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*.

A)



B)

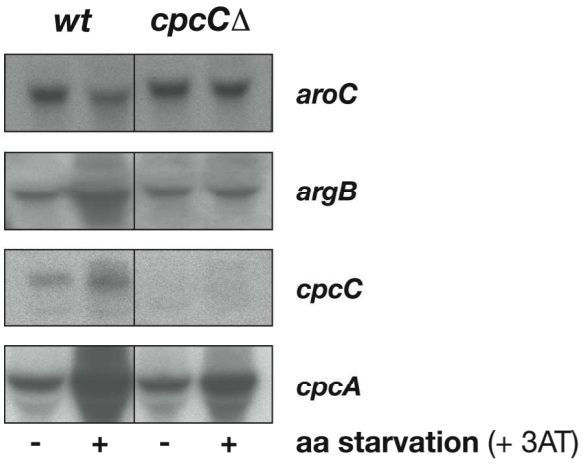


Figure 3.4. Phenotypes of a *ΔcpcC* mutant strain under amino acid starvation conditions
Strain AfS26 carrying the resistance marker was used for phenotypic characterisation, which behaved identical to strain AfS27 [*cpcC::loxP*]. A) Growth behaviour on minimal medium in the presence of glutamine starvation induced by MSX. For comparison, the clinical isolate D141 [*wt*] as well as the deletion strain AfS01 [*ΔcpcA*] 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 *ΔcpcC* 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.

3.4.4 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. 3.5A). In accordance with this result, enzymatic activities of the *argB*-encoded OTCase were elevated (fig. 3.5B). To our surprise, the elevated eIF2α-P signal was also clearly and reproducibly detectable from crude extracts that had been prepared from the *ΔcpcC* 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 *ΔcpcC* 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.

A)

B)

Figure 3.5. eIF2α phosphorylation is not abolished in a *ΔcpcC* background
A) Western experiments using crude extracts from strains D141 [*wild-type*] and AfS26 [*ΔcpcC*] 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 *ΔcpcC* strain to indicate that CpcC-specific phosphorylation of eIF2α is required for a proper Cross-Pathway Control response.

3.4.5 Virulence of *A. fumigatus* is not affected in a *ΔcpcC* background

In a previous study we had demonstrated that the transcriptional activator of the *A. fumigatus* CPC system is 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 *ΔcpcC* 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 killing of twelve mice of a 13-animal cohort in the experimental time frame (fig. 3.6). 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-typ*e and the *ΔcpcC* mutant background.

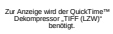


Figure 3.6. Virulence of *A. fumigatus ΔcpcC* strains is unaltered in a murine model of pulmonary aspergillosis. Survival plots for groups of leukopenic CD1 mice intranasally infected with $2x10^5$ conidia of *A. fumigatus* strains D141 [*wild-type*] (left panel) or *ΔcpcC* deletion mutants (right panel) 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.

3.5 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 *ΔcpcA* mutant had been carried out in the same model, making virulence characteristics of *A. fumigatus ΔcpcA* and *ΔcpcC* 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 *ΔcpcC* 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 *ΔcpcC* 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 *ΔcpcC* background: an *ΔifkB* mutant strain is not impaired in its CPC response and an *ΔifkB*; *ΔcpcC* 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, and we consider it unlikely that 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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Chapter 4

The Basal Proteome Directed by the *Aspergillus fumigatus* Transcriptional Regulator CpcA

4.1 Abstract

The opportunistic pathogenic fungus *A. fumigatus* requires for full virulence the transcriptional regulator CpcA, as a deletion of this gene leads to reduced virulence. However, the sensor kinase CpcC is not required for infection indicating that the basal expression level of CpcA is sufficient to support pathogenicity. In this study a part of the basal CPCome *via* proteomic experiments was analysed by comparing the *wild-type* D141 isolate with its *ΔcpcA* deletion derivative under sated conditions. By DIGE analysis 22 regulated proteins with more than a 1.4 fold induction/repression could be identified, with 16 of these being increased in the *wild-type* and eight showing a decreased amount compared to the deletion strain. Classification *via* annotation made it possible to place them in different groups of function. Three proteins were identified that may be involved in pathogenicity of *A. fumigatus*, e.g. the Asp hemolysine. Furthermore proteins needed for metabolism, which form the largest group, and one protein having the function to neutralise reactive oxidative species were also found. With the aim to identify putative CpcA binding sites the promoter regions of the encoding genes were investigated. Four typical sequences known as putative recognition sites of the transcriptional factor were checked and found in eight genes of the regulated proteins. These data shed first light on the basal CPCome, which is necessary but also sufficient to support pulmonary aspergillosis.

4.2 Introduction

The air borne filamentous fungus *A. fumigatus* has been recognised to be one of the most common organisms to evoke invasive aspergillosis (Soubani and Chandrasekar, 2002). By producing conidia with a size about 2-3 µm in diameter the fungus has the ability to reach easily the lung aveoli of the host (Latgé, 1999); (Brakhage and Langfelder, 2002). Moreover, its pronounced thermotolerance with an optimal growth temperature at 37°C and a survival up to 55°C makes it possible that *A. fumigatus* can grow very well in mammalians (Chang *et al.*, 2004; Cooney and Emerson, 1964; Maheshwari *et al*., 2000). Like other pathogenic organisms the fungus needs special abilities to survive in the host whereas limited nutrients and the immune defence system are the main barriers, which must be overwhelmed. Incoming spores are attacked by macrophages and neutrophils as primary defence response of the host. These phagocytosing cells induce oxidative stress by using reactive oxidant intermediates (ROI) to kill the spores (Philippe *et al.*, 2003). Therefore, defence mechanisms as the mitogen-activated kinases (MAPKs), which constitute a regulation system, that enables the fungus the adaptation to environmental stress like neutralisation of oxidative molecules are required. Four different proteins of the MAPKs are found in *A. fumigatus* called SakA, MpkA, MpkB and MpkC (May *et al.*, 2005). Other enzymes like glutathione transferases are needed for inactivation of reactive oxidative species (ROS) as well. In *A. fumigatus* there are three of these enzymes that show a peroxidase activity, by which the fungus has the possibility to detoxify of oxidative intermediates (Burns *et al.*, 2005).

As mentioned above the ability of *A. fumigatus* to respond on the defence mechanism of the host is as important as the competency to get nutrients from the surroundings. The lung, which is the primary target of incoming spores, is a place where substrates and nutrients are complex and unbalanced. This organ consists mainly of proteins like collagen (Bromley and Donaldson, 1996; Gil *et al.*, 1996; Penalver *et al.*, 1996; Tronchin *et al.*, 1993) so that there is no abundance of free nitrogen and carbon sources. To grow under these circumstances degrading proteins and pathways for starvation conditions are indispensable for the fungus. Therefore proteases are necessary for the fragmentation of lung components into peptides. Afterwards up take systems consisting of oligopeptide transporters are involved to transport the nutrients into the fungus as described in earlier works for *C. albicans* (Reuss and Morschhäuser, 2006) whereas nitrogen and carbon become available for the organism. Not only proteases but also pathways to recognise the starvation conditions in the surrounding and to activate genes involved in metabolism like amino acid biosynthesis are important. One of these pathways for recognition nutrient limitation resulting in a regulation of different kinds

of genes in the fungus is the Cross Pathway Control (CPC), the homologous system to the General Control (GC) in *S.* cerevisiae (Hinnebusch, 1984; Hoffmann *et al.*, 2001; Krappmann *et al.*, 2004). The CPC consists of a sensor kinase called CpcC and a transcriptional regulator CpcA. The *cpcA* gene is regulated on transcriptional level and on translational level (Hoffmann *et al.*, 2001; Krappmann *et al.*, 2004). Under non-starvation conditions where nutrients are available for the fungus the *cpcA* translational level is repressed to result in a basal expression level of CpcA. Responsible for the repression is the high amount of unphosphorylated initiation factor for translation eIF2. This leads to a translation of the two small uORFs within the promoter region of the *cpcA* gene, which are translational regulatory elements. Under starvation conditions, uncharged tRNAs are recognised by CpcC leading to a phosphorylation of eIF2 to eIF2~P. As a result the coding sequence of CpcA is translated leading to an increased amount of this transcriptional activator (Braus *et al*., 2004). With more than 500 target genes CpcA belongs to the global regulators within the fungus. Transcriptomic analysis of the CPC/GC under starvation conditions was performed in different organisms like *S. cerevisiae*, *N. crassa* and *A. fumigatus* (Natarajan *et al.*, 2001; Tian *et al.*, 2007) and the comparison of these data show that there are a lot of similarities of the regulated group of genes. In all organisms amino acid biosynthesis genes are up-regulated as well as genes involved in carbon and nitrogen metabolism (Natarajan *et al.*, 2001; Tian *et al.*, 2007). Overall the CPC/GC give the different organisms the ability to react on changing environmental and nutritional conditions.

Earlier studies indicated a role for CpcA in pathogenicity of *A. fumigatus*. Deletion of the transcriptional regulator decreased the virulence of this fungus in a murine mouse model in comparison to the *wild-type* strain (Krappmann *et al.*, 2004). In contrast to that a *ΔcpcC* strain displays no reduction in virulence in immuno-compromised mice (see Chapter 2). *In vitro* and *in vivo* experiments with a *gfp::cpcA* construct displayed no induction of the CPC. These results lead to the suggestion, that the basal expression level of CpcA is sufficient for full virulence and that de-repression of CpcA expression is not required in invasive aspergillosis (Sasse *et al.*, 2008).

In this study *A. fumigatus* genes regulated in dependency of the basal CpcA expression level were identified by a proteome approach. The proteomic profiles of a *wild-type* isolate and its *ΔcpcA* deletion strain were compared under sated conditions to reveal an incomplete section of the basal CpcA-directed proteome of *A. fumigatus*.

4.3 Materials and Methods

4.3.1 Media, culture conditions and strains

The *wild-type* isolate D141 (Staib *et al*., 1980) and the *ΔcpcA* deletion strain AfS01 (Krappmann *et al*., 2004) were used in these experiments. Inoculation was made in liquid minimal medium supplemented according to Käfer (1977). As nitrogen source ammonium was used (10mM final concentration). Each strain was inoculated in 400 ml minimal medium with $2x10^6$ spores/ml and incubated at 37°C for 18 h. Mycelia were harvested by filtration through miracloth filter (CALBIOCHEM) washed twice with sterile water and subsequently ground in liquid nitrogen.

4.3.2 Protein extraction and determination of concentration

50 mg of ground mycelium was transferred to an Eppendorf reaction cup. Extraction was carried out by using the TCA/aceton precipitation method as described by Darmerval *et al*. (1986) with some modifications. The 50 mg of mycelium were incubated with 500 µl acetone/13.3% w/v trichloracetacid and 0.09% w/v DTT over night. After centrifugation at 14000 rpm for 15 min at 4°C the supernatant was discarded and the pellet was washed with 1 ml ice-cold acetone containing 0.009% w/v DTT. For removal the wash solution centrifugations were done. Afterwards the pellet was dried 5 min at RT. 300 µl lysis buffer [7 M urea, 2 M thiourea, 2% (w/v) CHAPS, 1% (w/v) Zwittergent 3–10, 0.8% (v/v) IPG buffer pH 3-11, 20 mM DTT, 20 mM Tris] described by Kniemeyer *et al*. (2006) with some modifications was added for resuspension the pellet and the received solution was incubated for 1 h at room temperature. The supernatant was collected after centrifugation at 14000 rpm at 16°C for 30 min. The protein concentration was determined according to the protocol of Bradford (1976). Therefore BIO Rad protein assay (BIO-RAD Lab., Hartfordshire, USA) was used.

4.3.3 2-D gel electrophoresis

For pre-experiments 2-D gel electrophoresis according to the protocol of Kniemeyer *et al*. (2006) was performed. In the experiments strips of 24 cm with a non-linear pH gradient of 3-11 (Healthcare Biosciences, GE) were used and rehydrated over night in 450 µl rehydration buffer [7 M urea, 2 M thiourea, 2% (w/v) CHAPS, 1% Zwittergent, 0.002% (w/v) bromophenolblue, 0.5% (v/v) IPG buffer, 1.2% (v/v) De-Streak Reagent (Olsson *et al*. 2002) (GE Healthcare Bio-Sciences)]. For the experiments 220 µg from each sample were used. For isoelectric focussing the IPGphor II with a manifold ceramic tray (GE Healthcare Bio-

Sciences) was used. The separation in the second dimension was realised on the Ettan DALT System (GE Healthcare Bio-Sciences) with lab cast 1.0 mm SDS polyacrylamide gels [12.5% (w/v)] containing Rhinohide as a gel strengthener (Molecular Probes, Leiden, NL): 20 mA/gel for minigels and 30 min 30 W, 4 h 100 W for six Ettan Dalt gels. Protein standard Mark 12 was purchased from INVITROGEN (Karlsruhe, Germany).

4.3.4 Visualisation of proteins by Coomassie Brilliant Blue staining (CBB)

Spots were visualised by colloidal CBB staining according to the protocol of Neuhoff *et al*. (1988). For fixation gels were incubated in fixing solution consisting of 40% (v/v) methanol, 7% (v/v) glacial acetic acid by incubation for two hours. Afterwards the gels were washed twice with ultrafiltrated water for 20 min and stained with staining solution containing 10% (w/v) methanol, 0.1% (w/v) CBB G-250, 1.6% (v/v) o-phosphoric acid and 10% (w/v) ammonium sulfate over night. Stained gels were neutralised by incubation in a solution containing 0.1 M Tris-base titrated with o-phosphoric acid to a pH at 6.5 and incubated for 5 min. Afterwards the gels were washed in 25% (v/v) methanol for 1 h and stored in the methanol solution at 4°C.

4.3.5 DIGE experiments, analysis and identification of spots

For DIGE experiments five gels were made by using two biological replicates. Samples of the wildtype and the deletion strain were prepared according to Kniemeyer *et al*. (2006) with some modifications as mentioned above. Afterward the samples were adjusted at a pH of 8.5 by addition of 100 mM NaOH. For labelling CyDye minimal dyes according to the manufacturer's protocol (GE Healthcare Bio-Sciences) were used. 50 µg protein of each sample were labelled with 300 pm of CyDye. Samples of both strains were labelled either with Cy5 or Cy3. A pool of both (*wild-type* and *ΔcpcA*) was labelled with Cy2 for internal standard. Samples were mixed and incubated in the dark on ice for 30 min. The reaction was stopped by adding 1 µl of 10 mM L-lysine. An equal volume of 4x sample buffer (same components as for the lysis buffer mentioned above, plus 3.2% [vol/vol] ampholytes and 40 mM dithiothreitol) was added. 150 µg of each labelled preparations were loaded for the first dimension on a ceramic tray by using the IPGphor II (see description above) using 24 cm stripes with a pH range from 3-11 (GE Healthcare Bio-Sciences). Gels were scanned by a Typhoon 9410 scanner using a resolution of 100 µm and analysed with the DeCyder software (GE Healthcare). Afterwards gels were stained with coomassie as mentioned above. Spots with a regulation with at least 1.4 fold were cut out by manual and digested with trypsine for

mass spectrometry described by Shevchenko *et al*. (1996). Tryptic peptides extracted from each gel slice were injected onto a reversed-phase liquid chromatograohic column (Dionex NAN75-15-03-C18 PM) by using a HPLC system from Dionex, Amsterdam, Netherlands. For mass analysis a LCQ DecaXP mass spectrometer (Thermo Electron Corp., San Jose) CA9 equipped with a nanoelectrospray ion source was used. Annotation was done by using the database of TIGR.

4.4 Results and Discussion

Previous works had demonstrated the involvement of the transcription factor CpcA in pathogenicity (Krappmann *et al.*, 2004). In addition, data had been presented leading to the suggestion that the basal expression level of CpcA seems to be sufficient for pathogenicity, so that an induced CPC is not crucial for virulence of *A. fumigatus*. To identify the "basal proteome" generated by this transcriptional activator, the proteome of *wild-type* strain D141 was compared to the one of the *ΔcpcA* deletion strain AfS01 under non-starvation conditions.

4.4.1 Differentially expressed proteins in dependency of basal CpcA expression

Five independent multiplex DIGE experiments of two different biological replicates were performed, whereas one of these gels represents three normal gels resulting in 15 different kinds of comparable gels for this study. After matching and quantification of all gels by using the DeCyder software, 1574 spots could be detected that appeared to be induced. For restriction conditions a cut-off at 1.4 fold or higher was set for the regulated spots. In addition, only spots that show modulation in at least 12 of the 15 samples were used for further investigation. Also signals with a t-value higher than 0.05 or without any value were not considered in our studies. 64 regulated spots remained after this restrictive procedure, but 41 of them were not picked because their signals were too weak after a Coomassie staining or the spots could not be clearly localised, so in the end 23 differentially regulated signals were cut out from the gels for mass spectrometry analysis (fig. 4.1). Analysis of these extracted putative gene products retrieved 22 different proteins, with one of them being found twice (spot numbers 1327 and 1329). In this data set only seven spots display an increased expression level in the *ΔcpcA* strain; the remaining 16 spots showed a higher level in the *wild-type* strain. All detected and analysed proteins have no higher amount than 1.81 fold (table 4.1). Altogether it can be said that the picked and analysed spots from this data set show only a modest regulation, however as a result of the restriction conditions it can be assumed that these proteins are part of the basal CPC proteome.

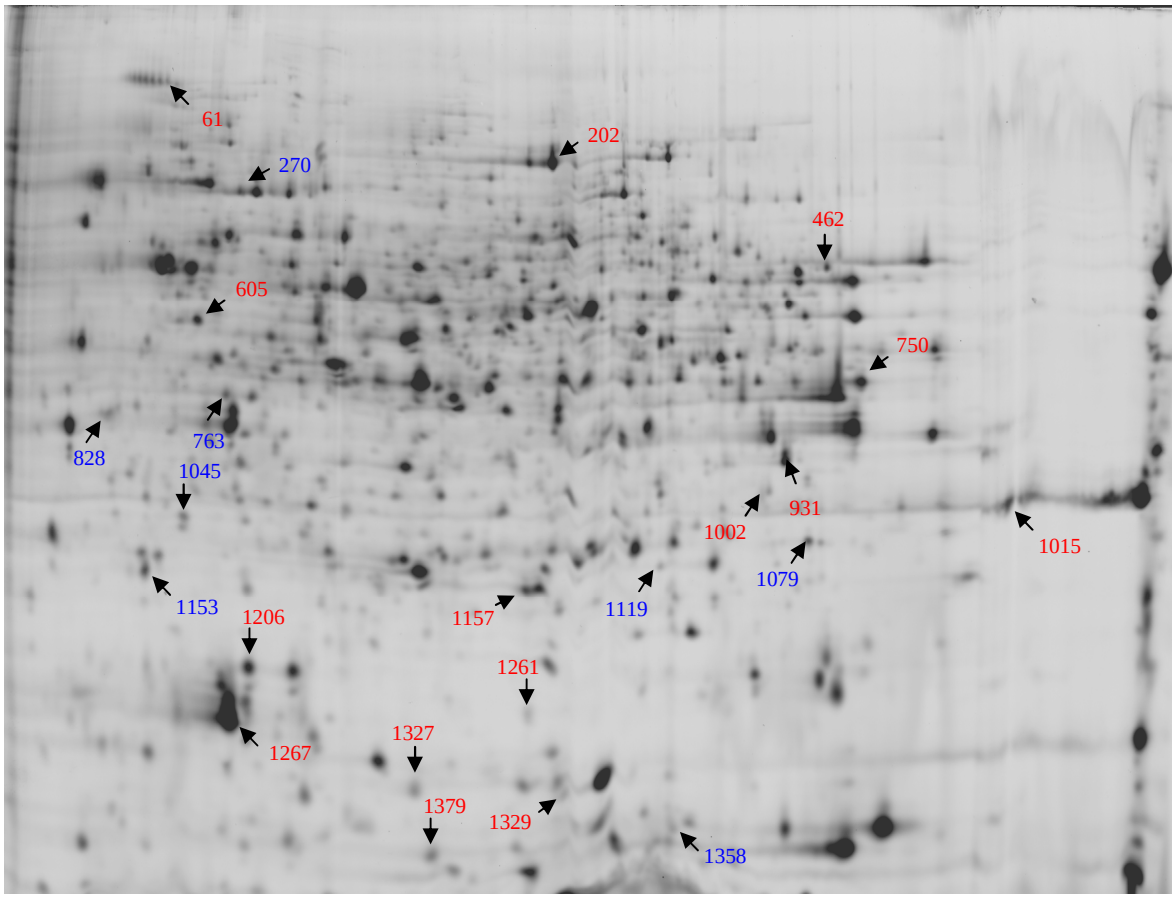

Figure 4.1. Detected spots from the DIGE after staining with Coomassie Brilliant Blue.
The figure shows all 23 detected and analysed proteins. The arrow shows the corresponding spots of every number. Up-regulated proteins of the *wildtype* in comparison to the *cpcA* deletion strain have red coloured numbers and down regulated are displayed by blue numbers.

Table 4.1. Detected and identified proteins

Detected protein	**Pos.**	**Av. Ratio***
Afu4g12450 pH domain protein	61	1.81
Afu5g01440 allergen putative	1329	1.70
Afu5g06240 alcohol dehydrogenase	750	1.65
Afu3g11710 saccharopine dehydrogenase, putative	605	1.60
Afu7g05660 elongation factor EF-3, putative	202	1.60
Afu2g03140 peptide methionine sulfoxide reductase	1261	1.56
Afu1g09930 glycerol dehydrogenase Gcy1	931	1.53
Afu6g02280 allergen Asp F3	1267	1.53
Afu8g05320 mitochondrial F1 ATPase subunit alpha	462	1.51
Afu3g00590 Aegerolysin family protein, identical to Asp hemolysin	1379	1.50
Afu6g10260 aldehyde reductase (AKR1)	1157	1.48
Afu6g11620 formyltetrahydrofolate deformylase	1002	1.41
Afu4g06910 outer mitochondrial membrane protein porin	1015	1.45

Table 4.1 continued.

Detected protein	Pos.	Av. Ratio*
Afu2g03010 cytochrome c subunit	1206	1.44
Afu1g16840 TCTP domain protein	1153	-1.41
Afu8g03930 Hsp70 chaperone (HscA)	270	-1.42
Afu6g06450 proteasome component Pre4	1119	-1.44
Afu1g05080 ribosomal protein P0	763	-1.45
Afu6g06750 14-3-3 family protein, similar to 14-3-3 protein important for polarized growth	828	-1.50
Afu3g00730 conserved hypothetical protein	1045	-1.60
Afu5g08830 HEX1	1079	-1.71
Afu3g07430 peptidyl-prolyl cis-trans isomerase	1358	-1.72

*multiplication factor for regulation

4.4.2 Groups of function of the basal proteome directed by CpcA

The complete set of identified proteins was classified and divided into different groups of function. Analysis of the 14 proteins that are up regulated in the *wild-type* leads to a preliminary categorisation in seven different classes (table 4.2). Most gene products of these analysed proteins belong to metabolic pathways. For example the alcohol dehydrogenase (Afu6g102690), an enzyme involved in fermentation, that was 1.65 fold increased in the *wild-type* situation. Other proteins were identified, one with locus tag Afu1g09930 encoding a glycerol dehydrogenase that showed an up-regulation of 1.53 fold in the D141 strain. Adjacent to the glycerol metabolism this protein seems to be also involved in stress response induced by salt concentrations. An aldehyde reductase (Afu6g10260) that had a 1.48 fold increased expression level was also detectable in the data as well as a formyltetrahydrofolate deformylase (Afu6g11620), which is likely required for synthesis of purine ribonucleotides. Surprisingly, just one enzyme that is involved in amino acid biosynthesis was found; this putive saccharopine dehydrogenase with the locus tag Afu3g11710 catalyses the final synthesis step for lysine.

The second largest group containing three proteins from the up-regulated data set belongs to the aerobic respiration system comprising the proteins with the locus tags Afu2g03010, Afu4g06910 and Afu8g05320. All three can be found in the mitochondrial membranes. The mitochondrial F1 ATPase subunit alpha is needed for ATP synthesis coupled with proton transport and therefore has just an indirect connection to the aerobic respiration system. The other two candidates, an outer mitochondrial membrane protein porin and the cytochrome c subunit Vb, are directly involved in the respiration system. Furthermore, proteins were found

that might play a role in pathogenicity of *A. fumigatus*. One of them is the allergen Asp3 for which it is known that it is a factor related to virulence. The other one is a putative allergen, for which the function in connection with virulence is not really clear. For both gene products it is known that they are needed in the regulation of cell redox homeostasis. The third one is the Asp-hemolysin. In earlier studies it was shown that hemolysin supports the infection by *A. fumigatus* in a mouse model and that antibodies against this factor can decrease infection in an animal model. Nevertheless, it is not a main virulence factor but it may support other proteins involved in pathogenicity (Malicev *et al.*, 2007). The Asp-hemolysin displays an up-regulation of 1.5 fold in comparison to the *ΔcpcA* deletion strain, leading to the suggestion that CpcA is necessary for expression of this protein under non-starvation conditions.

Another important protein that was found with an increased level of 1.56 fold paticipates to the reaction on oxidative stress. The corresponding locus Afu2g03140 designates a gene that encodes a putative peptide methionine sulfoxide reductase, which may be involved in the reaction on oxidative stress. Experiments in *S. cerevisae* had pointed out that methionine sulfoxide reductases have the ability to reverse methione oxidation (MetO) caused by reactive oxidative species (ROS) (Moskovitz *et al.*, 2000; Moskovitz *et al.*, 2002). A dysfunction of these proteins leads to an accumulation of carbonyl proteins and results in a higher aging in yeast cells (Oien and Moskovitz, 2007).

The last two proteins, which were identified in our studies, are a putative elongation factor (Afu7g05660) that is needed for translation and a pH domain-containing protein (Afu4g12450), which may act as a kind of chaperone or may be important to react on changing pH conditions.

From the eight analysed spots displaying a down-regulation in the *wild-type,* five of them were classified and assigned to functional categories (table 4.2). Three are hypothetical proteins or proteins with unknown function so that a disposition in a special functional category was not possible. In addition the heat shock protein Hsp70 chaperone (HscA) having the locus tag Afu8g03930 was identified. From yeast it is known that the homologue Ssb2p interacts with the Hsp70 family member Ssz1p and the J-domain protein Zuo1p. These three chaperones form a functional triad required for translation. Deletion of one of these components leads to different dysfunctions and to a reduced growth in *S. cerevisiae* (Conz *et al.*, 2007; Gautschi *et al.*, 2002; Hundley *et al.*, 2002). Other chaperones were not found in our studies. Proteins involved in protein metabolism and anabolism were also found in this proteomic experiments: the proteasome component Pre4, a subunit of the 20S proteasome concerned in the ubiquitin dependent protein catabolism displays down-regulation in the *wild-*

type. Moreover, a peptidyl-prolyl cis-trans isomerase putatively involved in protein metabolism was identified. Only one spot was identified as a protein involved in signal transduction and which belongs to the 14-3-3 family. These are dimeric, acidic proteins having an average size of 30 kDa that are highly conserved and can be found in all investigated eukaryotes. The number of these proteins differs from organism to organism and can range from one up to thirteen isoforms within one organism. In *S. cerevisiae* and *S. pombe* for example only two members of 14-3-3 family are described, whereas in *A. thaliana* thirteen of these proteins exist (van Heusden and Steensma, 2006). In contrast to this the pathogenic yeast *C. albicans* contains only one of these proteins (Cognetti *et al.*, 2002). Their function is very manifold so that the 14-3-3 proteins play a role in many different mechanisms and pathways resulting in many different interaction partners. The involvement in DNA repair, which was shown by Ford *et al*. (1994), is only one example. In *C. albicans* the single isoform of 14-3-3 proteins is important for filamentous growth and therefore for pathogenicity. Without the corresponding gene an invasive growth seems to be impossible for this organism (Cognetti *et al.*, 2002; Hurtado and Rachubinski, 2002). As a result of this complexity the research and analysis of this family is very challenging.

Table 4.2. Classification of the different proteins found in the DIGE experiments

Classification	**Av. Ratio**
metabolism	
Afu6g10260 aldehyde reductase (AKR1)	1.48
Afu3g11710 saccharopine dehydrogenase, putative	1.60
Afu1g09930 glycerol dehydrogenase Gcy1	1.53
Afu5g06240 alcohol dehydrogenase	1.65
Afu6g11620 formyltetrahydrofolate deformylase	1.41
aerobic respiration	
Afu2g03010 cytochrome c subunit	1.44
Afu4g06910 outer mitochondrial membrane protein porin	1.45
Afu8g05320 mitochondrial F1 ATPase subunit alpha	1.51
oxidative stress	
Afu2g03140 peptide methionine sulfoxide reductase	1.56
pH regulation	
Afu4g12450 PH domain protein	1.81
pathogenesis	
Afu3g00590 Aegerolysin family protein, identical to Asp hemolysin	1.50
Afu5g01440 allergen putative	1.70
Afu6g02280 allergen Asp F3	1.57

Table 4.2 continued.

Classification	Av. Ratio
translation	
Afu7g05660 elongation factor EF-3, putative	1.60
heat shock proteins	
Afu8g03930 Hsp70 chaperone (HscA)	-1.42
translation	
Afu1g05080 ribosomal protein P0	-1.45
signal transduction	
Afu6g06750 14-3-3 family protein, similar to 14-3-3 protein important for polarized growth;	-1.50
protein degradation and metabolism	
Afu3g07430 peptidyl-prolyl cis-trans isomerase	-1.72
Afu6g06450 proteasome component Pre4	-1.44
unknown function	
Afu3g00730 conserved hypothetical protein	-1.60
Afu1g16840 TCTP domain protein	-1.41
Afu5g08830 HEX1	-1.71

In summary in this particular study a variety of different proteins were found, which do not belong to one special functional group. In a next step it should be validated whether these proteins are direct targets of CpcA. For this purpose an analysis of the 5' upstream region was made for each of the 22 detected genes with the aim to reveal putative CpcA binding sites.

4.4.3 Presence of putative CpcA binding sites

CpcA belongs to the leucine zipper (bZIP) family of transcriptional activators and has the ability to bind to specific recognition sites called GCREs or CPREs (**G**eneral **C**ontrol or **C**ross-**P**athway Control **R**ecognition **E**lements). The basic region of these proteins consists of a pair α helices required for binding to the target DNA (Landschulz *et al.*, 1988; Struhl, 1989). For Gcn4p in *S. cerevisiae* it was shown that there is a high affinity to the canonical sequence 5'-TGACTCA-3' leading to strong binding that results in a direct regulation of the corresponding gene (Arndt and Fink, 1986; Hinnebusch, 1984; Natarajan *et al.*, 2001; Thireos *et al.*, 1984). This sequence as the recognition site of Gcn4p is highly conserved and can be found in other organisms like filamentous fungi. In *A. nidulans* this recognition sequence was detected and identified as a binding site for CpcA, the homologue of Gcn4p (Hoffmann *et al.*, 2001). It was possible to find this sequence in the promoter region of the *cpcA* gene. Studies in other filamentous fungi like *N. crassa*, for which also this recognition sequence was found in the 5' upstream region of the *GCN4*-corresponding gene *cpc-1*, had shown that this

sequence enables an autoregulation of the transcriptional regulator (Paluh *et al.*, 1988). Later, the same was shown for *A. nidulans* (Hoffmann *et al.*, 2001) leading to the suggestion that this autoregulation is also the case in *A. fumigatus* because there is the same recognition sequence in the *cpcA* promoter. Further detailed studies in *N. crassa* and *S. cerevisiae* had displayed that small variations in the canonical binding site also support interaction with the transcriptional activator CPC-1/Gcn4p. Given the high similarity between CpcA and its homologues in the other organisms it is not surprisingly that these short sequences are also detectable in *A. fumigatus*, where they represent putative binding targets for the *cpcA* gene product. In this study the promoter regions within 1000 basepairs of genes corresponding to the proteins detected in the proteomic analysis were analysed for presence of the canonical sequence 5'-TGACTCA-3' and three further putative binding sites (fig. 4.2). Although the affinity of the transcriptional activator to these three diverging recognition elements is not as high as to the original one, previous works showed that the possibility of binding is still increased in contrast to other sequences (Tian *et al.*, 2007).

Figure 4.2. Putative CpcA binding sites
Investigation of the promoter region within 1000 basepairs for putative CpcA binding sites of the analysed proteins. Four target sequences were checked. The first one is the canonical sequence with the highest affinity to CpcA.

Investigation of the promoter regions of the genes resulting from the corresponding proteins of the proteomic data set yielded to the result that eight genes have one of the four checked sequences within their promoter region (table 4.3). Five of them are up-regulated proteins in the data set and three are down-regulated. Only one of the five up-regulated genes which encodes the putative allergen with the locus tag Afu5g01440 contains the high affinity binding site 5'-TGACTCA-3', all others have the less specific binding sequences in their promoter regions. In three upstream regions the 5'-TGAgTgA-3' sequence was found containing two mismatches from C to G at the fourth and sixth positions. The gene with the locus tag Afu3g11710 encoding the saccharopine dehydrogenase is the only protein having in its promoter the sequence 5'-TGAgTCA-3' as putative binding region for CpcA. The sequence 5'-TGACTgA-3' does not appear in any of these genes. In all three down-regulated

genes the typical CpcA binding region is missing whereas the sequence 5'-TGAgTCA-3' and once 5'-TGACTgA-3' was detected twice. Promoter regions with two or more regulation elements could not be found.

Table 4.3. Proteins of the proteomic experiments containing a putative CpcA binding site

Gene	CPRE
Afu3g11710 saccharopine dehydrogenase, putative	5'-TGAGTCA-3'
Afu6g11620 formyltetrahydrofolate deformylase	5'-TGAGTGA-3'
Afu3g00590 Aegerolysin family protein, identical to Asp hemolysin	5'-TGAGTGA-3'
Afu5g01440 allergen putative	5'-TGACTCA-3'
Afu7g05660 elongation factor EF-3, putative	5'-TGAGTGA-3'
Afu8g03930 Hsp70 chaperone (HscA)	5'-TGACTGA-3'
Afu3g00730 conserved hypothetical protein	5'-TGAGTCA-3'
Afu1g16840 TCTP domain protein	5'-TGAGTCA-3'

Finally, a comparison of transcriptomic data monitored for the transcriptional activator CpcA under amino acid starvation conditions (see Chapter 1) with the proteomic data set was carried out to detect proteins, which are also regulated on transcriptional level under starvation conditions. Only two proteins could be found, which were regulated in both experiments: the aldehyde reductase (AKR1) and the putative saccharopine dehydrogenase, both involved in metabolism.

To summarize these results it can be stated that proteins involved in different cellular functions depend in their expression level under non-starvation conditions on the CPC activator CpcA. However, their significance for pathogenicity of *A. fumigatus*, especially with respect to the necessity of the Cross-Pathway Control transcriptional activator CpcA, remains to be demonstrated. A prime candidate for further research is the Asp hemolysin-encoding gene. For this, a significant dependency of expression on CpcA is evident and the corresponding promoter region comprises a conserved, high affinity binding site for this activator. Accordingly, absence of CpcA results in reduced levels of Asp hemolysin due to the fact that no binding and activation at the promoter of the encoding gene occurs. Yet, further confirmatory studies will have to be carried out in order to substantiate this.

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Chapter 5

Conclusions & Outlook

5.1 Conclusions & Outlook

Pathogenic organisms like the filamentous fungi *A. fumigatus* require pathways and mechanisms for adaptation within the host. A better understanding of these regulatory systems make easier to find new targets for antifungal drugs and pharmaceutica. One of these systems is the Cross-Pathway Control (CPC), which is the homologous to the well-studied General Control of amino acid biosynthesis (GC) (Deng *et al.*, 2004; Hannig and Hinnebusch, 1988; Hannig *et al.*, 1990; Hinnebusch, 2005). In its core, this global regulatory system contains a sensor kinase (CpcC) and a transcriptional regulator (CpcA) (Hoffmann *et al.*, 2001), for which it was demonstrated that it is required for full virulence of *A. fumigatus* (Krappmann *et al.*, 2004). Therefore, the main focus of this work was set on the CPC with its components CpcC and CpcA. For sure a better understanding of this regulatory mechanism enables a more detail insight of a pathogenic filamentous fungus resulting in increasing knowledge that can give hints for new therapies against this pathogen.

5.1.1 The CPC is a global network required for many different stress responses

In the first part of this work it was the aim to find out what kind of genes are under the regulation of the transcriptional regulator CpcA and so are parts of the CPC regulatory network. Microarray hybridisation experiments were carried out as experimental approach to get a comprehensive overview. 523 putative CpcA targets with a regulated mRNA level of at least 1.5 fold were detected, with 377 genes being up-regulated in this data set and only 146 being repressed under the applied conditions (for an overview see fig. 5.2). By the finding that several transcriptional regulators are induced under starvation it becomes clear that the Cross-Pathway Control is a global regulatory system acting on additional factors and regulators to produce a fast and strong response to incoming environmental signals. It could also be confirmed that the CPC is involved in the regulation of different metabolism pathways as it has been shown for other fungi like yeast or *N. crassa* (Natarajan *et al.*, 2001; Tian *et al.*, 2007). In addition to many amino acid biosynthesis genes also a high number of transporters and permeases are up-regulated in our data set. Some of them are not required for metabolism but involved in pumping toxic molecules out of the fungus. This illustrated the requirement of

the system for detoxification of different drugs by channelling them *via* transport mechanisms out of the organism and not only to react on starvation condition like amino acid limitation or glucose and purine starvation (Rolfes and Hinnebusch, 1993; Yang *et al.*, 2000). The detection of putative stress response genes involved in neutralisation of oxidative reactive species and others, which confer the fungus a higher resistance to different kind of drugs e.g. itraconazole corroborates the suggestion that the CPC control is an important stress response system acting widely on the cellular level. Additionally, two genes encoding proteins of the AAA-ATPases family (*cdaA* and *cdaB*) could be identified on which our further experiments were based. The family of the AAA-ATPases (**A**TPases **a**ssociated with various cellular **a**ctivities) can be found in all kingdoms and show a diversity of functions in every organism. The core of the AAA proteins is an ATP- binding domain, which contains about 200 to 250 amino acids. In earlier studies it was possible to localise the different parts of the AAA proteins by crystal structure analysis and to detect oligomers of AAA ATPases, which normally occurs in hexamers (Hanson and Whiteheart, 2005). In general the proteins have six different domains, which give them the typical characterisation. Most of these proteins undergo a conformation and or bind ATP for hydrolysation resulting in physical works. But the exactly mechanism of changing the conformation is still not completely understand. The functions of these proteins range from reactions during replication *via* protein folding and degradation to processes in which chaperones are involved. But till now the function of many AAA-ATPases is still unclear and more effort will be needed to get a better and more understandable overview of this group of proteins (Davey *et al*., 2002; Hanson and Whiteheart, 2005; Wang *et al.*, 2004). Analysing genomic databases of different organisms showed that most of the eukaryotes contain 50-80 members of this family. *Arabidopsis thaliana* is an exception with a number of circa 140 AAA-ATPases. Resulting from this the interest was to find out how many of these proteins are exist in *A. fumigatus via* blasting the AAA-motif sequence against the genome of this fungus, by which only eleven putative proteins were retrieved.

After validation *via* Northern hybridisation that CpcA regulates both *cda* genes their cellular function was investigated by phenotypic characterisation of deletion mutant strains. From this, a putative involvement in resistance to conditions evoking cell wall stress was deduced. In addition, induction of CpcA by Calcofluor White treatment but not activation of the whole CPC system was evident. Therefore, it can be suggested that CpcA as the CPC transcriptional regulator can also be induced independently from this global system *via* different stress signals. Another hint for this suspicion is given by comparison of the amino acid starvation

data set with the transcriptional profile of *A. fumigatus* under antifungal stress induced by voriconazole (da Silva Ferreira *et al.*, 2006). After 4 h incubation with voriconazole the data set show an increased transcriptional level of CpcA as well as the two *cda*-encoded AAA-ATPases but not of typical amino acid biosynthesis genes like *argB*, which would indicate an induction of the CPC. Interestingly, no induction of the other nine AAA-ATPases in either of these data sets was evident. Therefore, it can be assumed that these two AAA-ATPases (CdaA and CdaB) are the only genes of this family regulated by CpcA. It will be interesting for further studies to analyse the functions of these proteins in more detail. In addition, analysis of other putative stress genes from the amino acid starvation data would be very interesting, especially in relation to drug response and reaction to oxidative stress.

5.1.2 The CPC is not triggered during the beginning of infection

Previous works had supported a role of CpcA in *A. fumigatus* pathogenicity (Krappmann *et al.*, 2004). Based on this finding it was the aim of this work to gain more information on this transcriptional activator during infection. A *gfp::cpcA* reporter strain was constructed to carry out *in vitro* confrontations with macrophages isolated from cell lines and mice. Interactions between these immune defence cells and conidia of *A. fumigatus* did not result in induction of CpcA expression, and additional *in vivo* experiments supported this observation. Accordingly, these experiments demonstrate that during the first period of infection the CPC system is not triggered, leading to the suggestion that starvation conditions for e.g. amino acid or glucose limitation do not exist in the murine lung or respectively in macrophage phagosomes (as overview see fig. 5.1). This result is supported by earlier studies in which proteomic experiments of the human pathogenic yeast *C. albicans* show an increased level of Gcn4p during ingestion by neutrophils but not by macrophages (Fernandez-Arenas *et al.*, 2007).

Furthermore, the sensor kinase of *A. fumigatus* required for the regulation of CpcA expression was characterised in detail. The function and the mechanism of this protein were described in earlier works for *S. cerevisiae*, *N. crassa* and *A. nidulans* (Carsiotis *et al.*, 1974; Davis, 2000; Hinnebusch, 1984, 2005; Hoffmann *et al.*, 2001; Piotrowska, 1980; Sachs, 1996). It found out that the deletions of CpcC leads to an intermediate growth phenotype in comparison to the *wild-type* and the *cpcA* deletion strain on medium inducing amino acid starvation and that CpcC is needed for induction of the CPC in *A. fumigatus*. Interestingly, a CpcC deletion strain is still able to phosphorylate the eIF2α subunit under amino acid starvation. The conclusion on this is that there might be another kinase with the ability to phosphorylate the initiation factor. However, it could be demonstrated that this residual phosphorylation has no effect on

CPC induction by increased CpcA translation. In further experiments the influence of CpcC on fungal pathogenicity was assessed in a murine model. The results made clearly demonstrated that CpcC, in contrast to CpcA, is not required for pathogenicity. Concluding from this and from the described microscopy experiments it can be suggested that the basal level of CpcA is sufficient for full virulence. Therefore it will be of interest to identify genes that depend in their transcription on the basal expression level of CpcA in order to get a better understanding for the infection mechanisms of this pathogenic fungus.

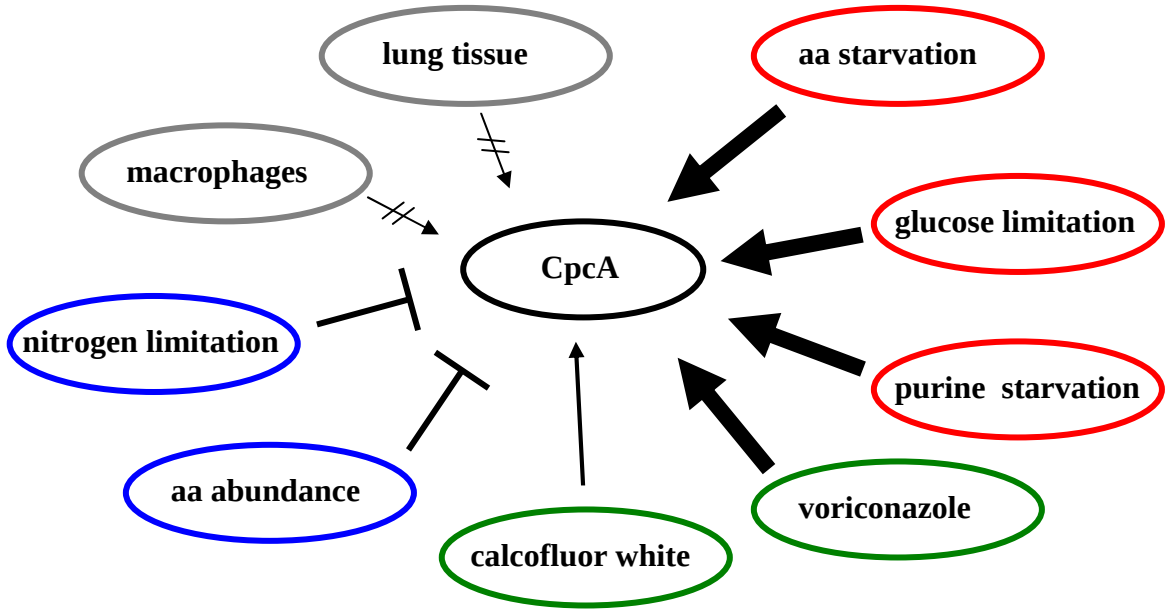

Figure 5.1. Different factors that influence the transcriptional activator CpcA
The scheme displays all influences, which have more or less an influence on CpcA. Red-circled factors have an inductive effect on the transcriptional activator and on the CPC. In contrast to that are the green-circled influences that only induce CpcA but have no effect on the CPC. Big arrows present in the figure strong up-regulation of CpcA. Factors and influences that repress the induction (blue circled) or have no effect to CpcA (grey circled) are also shown in this figure.

5.1.3 The basal level of CpcA regulates genes involved in pathogenicity

Based on the experiments showing that in contrast to CpcA CpcC has no influence to the virulence of *A. fumigatus* (Krappmann *et al.*, 2005; Sasse *et al.*, 2008) it was suggested that the basal expression level of transcriptional activator appears to be sufficient. In order to get a first overview on genes depending in their expression on the presence of CpcA under non-starvation conditions, a proteomic approach was followed. 22 different spots could be detected and assigned to 22 proteins (fig. 5.2). Their functional categorisation displayed a strong diversity; nevertheless, it was possible to find three putative candidates that are likely to be involved in pathogenicity. We also discover a protein whose function is required for

detoxification of oxidative reactive species. These results are perhaps a hint for an explanation of the *A. fumigatus* virulence in dependency of CpcA. In additional works we investigate the promoter regions of all 22 detected genes to find putative CpcA binding sites.

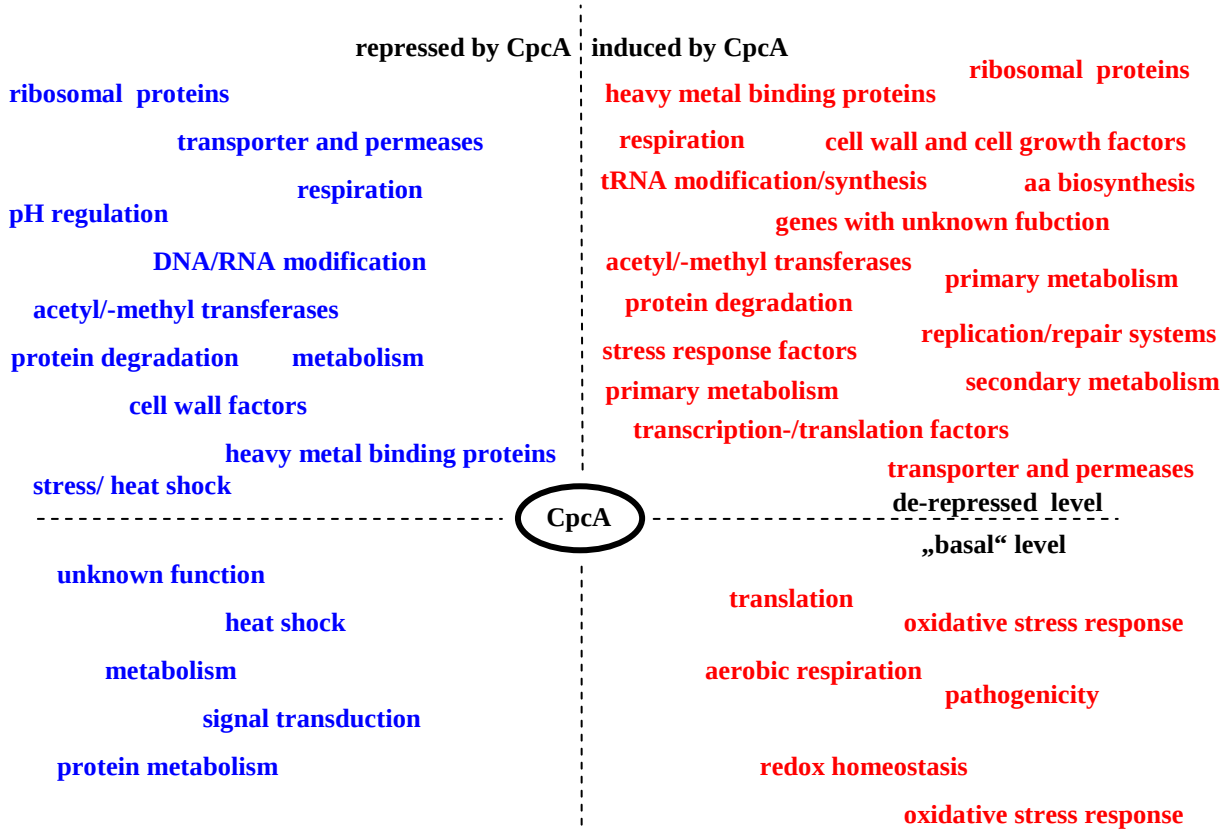

Figure 5.2. Groups of function that are regulated under starvation and non-starvation conditions in dependency of CpcA
The figure shows all main groups that are regulated by CpcA directly and indirectly. Red marked groups are here the induced ones and blue the repressed genes. The lower section in this figure represents the genes regulated under non starvation conditions hereas the upper part shows the targets of CpcA under amino acid starvation conditions.

Although some of these genes have binding sequence for the transcriptional regulator, surprisingly just two of the regulated proteins are present in the transcriptome data set on CpcA-regulated genes (see Chapter 1), and these have a function in metabolic routes but not in virulence. It can also be concluded from these results that this kind of basal regulation of CpcA-dependent genes is not as significant as under starvation conditions and that small differences in the amounts of some proteins are required to support pathogenicity of this fungus. To find out to what extent CpcA regulates these genes, further experiments like promoter analysis should be performed. In addition, deletion of target genes of CpcA under non-starvation condition may be a possibility to address the role of CpcA in pathogenicity.

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