AfuSomA transcriptional network in human pathogen *Aspergillus fumigatus*

Control of asexual development, adhesion and virulence

Dissertation

for the award of the degree

"**Doctor rerum naturalium"**

of the Georg-August-Universität Göttingen

within the doctoral program *Molecular Biology of Cells*

Of the Georg-August University School of Science (GAUSS)

Submitted by

Chi-Jan Lin

from Hsinchu/Taiwan

Göttingen 2014

Member of the Thesis Committee:

Prof. Dr. Gerhard H. Braus
Department of Molecular Microbiology and Genetics
Institute for Microbiology and Genetics

Prof. Dr. Stefanie Pöggeler
Department of Genetics of Eukaryotic Microorganisms
Institute for Microbiology and Genetics

Prof. Dr. Uwe Groß
Department of Medical Microbiology
Institute for Medical Microbiology

Members of the Examination Board:

Prof. Dr. Rolf Daniel
Department of Genomic and Applied Microbiology
Institute of Microbiology and Genetics

Prof. Dr. Kai Heimel
Department of Microbial Cell Biology
Institute of Microbiology and Genetics

PD Dr. Michael Hoppert
Department of General Microbiology
Institute of Microbiology and Genetics

Date of oral examination: 04.06.2014

Affirmation

I hereby declare that this thesis was written independently and with no other sources and aids than quoted.

Göttingen, 31.03.2014

Chi-Jan Lin

This work was accomplished in the group of Prof. Dr. Gerhard H. Braus, at the Department of Molecular Microbiology and Genetics at the Institute for Microbiology and Genetics, Georg-August University Göttingen

Parts of this work will be published in:

Chi-Jan Lin, Henriette Irmer, Oliver Valerius, Van Tuan Tran, Britta Herzog, and Gerhard H. Braus (2014). AfuSomA is required for adhesion, development and virulence of the human pathogen *Aspergillus fumigatus.* Manuscript in preparation.

Table of contents

Summary

Fungal development is regulated by environmental stimuli via various signaling pathways. For instance, the cyclic AMP dependent protein kinase A (PKA) cascade is controlling development or pathogenesis in fungi as filamentous growth in backer yeast *Saccharomyces cerevisiae* or pathogenesis in plant pathogenic fungus *Magnaporthe oryzae*. The cAMP/PKA downstream target Flo8/Som1 containing a conserved LUG/LUH-Flo8-single-stranded DNA binding (LUFS) domain is required for morphological development and virulence. In this study the *FLO8* homolog, *AfusomA* of the human opportunistic pathogenic fungus *Aspergillus fumigatus* was investigated. Heterologous expression of the AfuSomA encoding gene complemented invasive growth (cell-surface adhesion) or flocculation (cell-cell adhesion) in haploid or pseudohyphal growth in diploid ∆*flo8 S. cerevisiae* yeast strains. AfuSomA and ScFlo8 regulate the expression of the *ScFLO11* gene encoding flocculin as major component for adhesion by binding to similar regions on its promoter. These results indicate that AfuSomA and ScFlo8 share a similar function in yeast. Loss of the encoding AfuSomA resulted in a slow growth phenotype and a block in asexual development in *A. fumigatus*. Only aerial hyphae without further differentiation could be formed in *AfusomA* null mutant. An abolishment of conidiation was verified by a conditional expression of *AfusomA* using the inducible Tet-on system. Adherence to the host is an important step for pathogenesis. Adhesion assay with conditional expression strain indicated that AfuSomA is required for adherence to plastic surfaces. Infection of fertilized chicken eggs revealed that AfuSomA is required for pathogenicity. Transcription analysis showed that AfuSomA regulates expression of several transcription factors which have been shown to regulate conidiation and adhesion in *A. fumigatus*. GFP-Trap with AfuSomA leads to the identification of nucleolar proteins and PtaB (ScMfg1) which the yeast homolog forms complex with ScFlo8. Expression of putative adhesins was down regulated in *AfusomA* null strain. Single or multiple deletions of putative adhesins showed normal conidiation and pathogenicity.

Zusammenfassung

Die Pilz-Entwicklung wird durch Umwelt Stimuli über verschiedene Signal-Wege reguliert. So kontrolliert die zyklische AMP abhängige Protein Kinase A Kaskade die Entwicklung und Pathogenität in Pilzen, wie zum Beispiel in der Bäckerhefe *Sacharomyces cerevisiae* das filamentöse Wachstum oder im Pflanzen pathogenem Pilz *Magnaporthe oryzae* die Pathogenität. Das unterhalb der cAMP/PKA Kaskade angesiedelte Zielprotein Flo8/Som1 ist notwendig für morphologische Entwicklung und Virulenz und besitz eine konservierte LUG/LUH-Flo8-single-stranded DNA bindende Domäne (LUFS). In dieser Arbeit wurde das Flo8 homologe Protein AfuSomA des opportunistischen humanpathogenen Pilzes *Aspergillus fumigatus* näher untersucht. Dabei konnte gezeigt werden, dass die heterologe Expression von AfuSomA in *S. cerevisiae* Δ*flo8 Mutanten* invasives Wachstum (Zellen-Oberflächen Interaktion) und Flokkulation (Zellen Interaktion) wiederherstellt sowie in diploiden Mutanten Pseudohyphen Wachstum vermittelt. Beide, AfuSomA und ScFlo8 regulieren die Expression des Flocculins ScFLO11, einer Hauptkomponente für Adhesion, indem sie an ähnliche Promotor Bereiche binden. Aus den Ergebnissen ist zu schließen, dass AfuSomA und ScFlo8 eine ähnliche Funktion in Hefe übernehmen können. In *A. fumigatus* zeigte die Abwesenheit von AfuSomA ein langsames Wachstum und eine Blockade in der asexuellen Entwicklung. Nur Lufthyphen ohne jegliche Differenzierung wurden in der Null Mutante *AfusomA* gebildet. Zusätzliche wurde dieser Phaenotyp auch mittels des Tet-on induzierbaren Expressions-Systems in einer konditionalen Expressionsmutante bestätigt. Die Adhesion an den Wirt spielt eine wichtige Rolle in der Pathogenität von Krankheitserregern. Die Adhesionstudie der konditionalen Expressionsmutante zeigte, dass AfuSomA für die Adhesion an die Plastikoberfläche benötigt wird. Im Hühnerei-Infektionsmodell wurde der Einfluß von AfuSomA auf die Pathogenität nachgewiesen. AfuSomA reguliert verschiedene Transkriptionsfaktoren, die eine Rolle bei der Konidienbildung und Adhesion spielen. Dies wurde durch Transkriptionsanalysen gezeigt. Eine

Interaktion von AfuSomA mit nukleolaren Proteinen und dem aus Hefe bekannten Flo8 Interaktionspartner PtaB (ScMfg1) wurden mit dem "GFP-Trap" System gefunden. Weiterhin konnten putative Adhesine identifiziert werden, die durch AfuSomA reguliert werden. Die Deletionsmutanten dieser putative Adhesine zeigten normale Konidienbildung und Pathogenität.

1 Introduction

1.1 Pathogenic fungi

1.1.1 Fungal pathogens in plants and humans

The fungal kingdom contains roughly 1.5 to 5.1 million species living on various environments on this planet (Hawksworth and Rossman, 1997; Blackwell, 2011), and only 100.000 species are currently known. Among those fungi, only a small fraction are potential plant or human pathogens (Woolhouse and Gaunt, 2007; Hube, 2009; Robert and Casadevall, 2009; Gauthier and Keller, 2013). However, the limited fungal pathogens can cause enormous yield losses in agriculture and high costs in medical treatments together with loss of lives. Being pathogenic, fungi have to acquire nutrient in their hosts and in order to be successful they have to finish their life cycle which includes germination, growth, colonization and reproduction (Sexton and Howlett, 2006). To obtain the nutrients from the hosts, plant pathogens can be either biotrophic or hemibiotrophic. The difference is the latter feeds on living plants for a period of time and kills the hosts for gaining nutrients from dead tissues, whereas the biotrophic fungus completes its life cycle on living hosts (Giraldo and Valent, 2013). For example, *Magnaporthe oryzae* is a hemibiotrophic fungus causes yield losses of rice worldwide by destroying its host, while *Ustilago maydis* can live along with its hosts without its death. Most of human pathogens are environmental fungi, which normally live in soil or compost, and acquire nutrient from decaying material. The infection is acquired via the lung when their abundant airborne spores are inhaled. In contrast to commensal fungi as *Candida albicans* colonize the mucous surfaces of human as natural habitat which can become infectious when the immune system of host goes down (Hube, 2009).

The *Aspergillus* genus is comprised of filamentous fungi, which play a role in recycling carbon and nitrogen from decaying plant materials and are usually found in soil or compost. Among 260 different species in *Aspergilli* (Geiser *et al.*, 2007), *Aspergillus fumigatus* is the most common agent of human invasive fungal infections whose mortality rate in immunocompromised individuals is more than

60 % (Tekaia and Latgé, 2005; Gauthier and Keller, 2013). As a saprophyte, *A. fumigatus* usually can be found in various habitats and produces ubiquitous conidia (asexual spores), which can be easily dispersed into the air (Morris *et al.*, 2000). The infection process can start when the airborne conidia are inhaled by immunocompromised humans. The inhaled conidia in healthy individuals can be removed by pulmonary defenses, which include mucociliary clearance of epithelial cells and alveolar macrophages and leukocytes (neutrophils and eosinophils) in the lung (Dagenais and Keller, 2009; Lilly *et al.*, 2014). The conidia that evade from innate immunity germinate to form hyphae and penetrate into the barrier which is comprised of pulmonary epithelial cells and vascular endothelial cells between alveoli and blood vessels. The defense to this invasion is mainly mediated by neutrophils, dendritic cells and the adapted immunity. If the host is immunocompromised such as individuals with chronic pulmonary diseases or AIDS, this invasion is followed by dissemination of hyphal fragments via bloodstream and infection of deeper organs (Filler and Sheppard, 2006; Askew, 2008; Dagenais and Keller, 2009) (Figure 1).

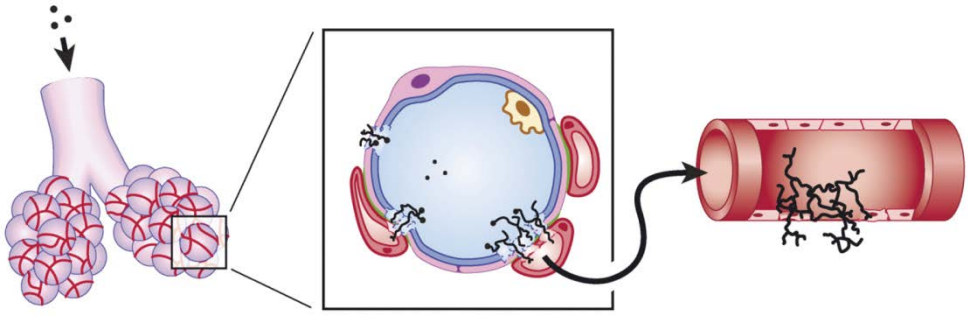


Figure 1. Model of pathogenesis in *Aspergillus fumigatus*.

Infection of *A. fumigatus* occurs when the inhaled conidia reach the alveoli in lung (left panel). The conidia can evade from the immune system and be able to germinate and damage the barrier between alveolus and an adjacent blood vessel (central panel). This barrier is comprised of pulmonary epithelial cells (pink) and vascular endothelial cells (red). Hyphal fragments which penetrate the barrier disseminate via bloodstream, adhere to the luminal endothelial cells and invade to deeper organs (right panel) (adapted from (Askew, 2008)).

Most of fungal pathogens can only infect either plants or animals, however several species from ascomycetes are able to cause diseases in both kingdoms (Gauthier and Keller, 2013). This indicates that some mechanisms are specific for plant or animal infection, while others are conserved in fungal pathogens and are required for general pathogenesis. For example, plant pathogens secrete cutinases and cellulases to hydrolyze cutin and cellulose during infection, while these hydrolytic enzymes are not required in pathogenesis in human pathogens. In *Fusarium oxysporum* which can infect both plant and animal, the protein kinase Fmk1 which regulates surface hydrophobicity and root attachment (Di Pietro *et al.*, 2001), is required for virulence in tomatoes but not in mice. In contrast, the transcription factor PacC, which controls pH homeostasis (Caracuel *et al.*, 2003), is essential for mice infection but not for tomato infection (Ortoneda *et al.*, 2004). Apart from the host specific virulence factors, some conserved mechanisms within different species are required for pathogenesis. In general organism needs a system that senses and transfers signals for nutrition, stress and environmental changes into cellular processes. The signal transduction pathway is a good example for a mechanism that is involved in pathogenicity.

1.1.2 Signal transduction: the heterotrimeric G protein and downstream cAMP/PKA pathway

The cyclic adenosine monophosphate (cAMP) dependent pathway is highly conserved from bacteria to mammals and plays an important role in pathogenesis in bacteria and fungi (McDonough and Rodriguez, 2012; Gancedo, 2013). It has been shown that the cAMP dependent protein kinase A (PKA) signaling pathway plays a major role in morphological development and virulence in plant and animals for fungal pathogens as *C. albicans*, *Cryptococcus neoformans*, *M. oryzae* or *U. maydis* (Mitchell and Dean, 1995; Durrenberger *et al.*, 1998; Hogan and Sundstrom, 2009; Kozubowski *et al.*, 2009; Ramanujam and Naqvi, 2010; Fuller and Rhodes, 2012). In eukaryotic cells, the cAMP/PKA signaling pathway begins with the heterotrimeric G protein. The G protein is

comprised of α, β and γ subunits and this complex is usually associated with a G protein coupled receptor (GPCR) which contains seven trans membrane α helix structures (Li *et al.*, 2007). Ligand bound GPCR replaces the GTP for GDP on the Gα subunit and liberates Gα protein from Gβγ dimer. Both Gα and the Gβγ dimer can regulate downstream signaling molecules which include adenylyl cyclases, phospholipases, phosphodiesterases, lipid kinases and ion channels (Neves *et al.*, 2002; Dorsam and Gutkind, 2007). The cAMP/PKA signaling pathway is downstream of adenylyl cyclase which is activated by the G protein.

The activated adenylyl cyclases convert ATP to cAMP. As a secondary messenger, cAMP binds to the regulatory subunits of PKA. The catalytic subunits of the enzyme are released and activate downstream transcription factors by phosphorylation (McDonough and Rodriguez, 2012). In the budding yeast *Saccharomyces cerevisiae*, the role of this pathway is nutrient sensing and regulates pseudohyphal growth as well as adhesion (Cullen and Sprague, 2012). In the opportunistic human pathogenic fungus *C. albicans*, this pathway is also activated by nutrient starvation and controls yeast to hyphae transition which is important for virulence (Hogan and Sundstrom, 2009; Inglis and Sherlock, 2013; Mayer *et al.*, 2013). This is also the case in the plant pathogen *U. maydis*, where dimorphic transition from yeast to filaments is activated by the cAMP/PKA pathway (Bölker, 2001; Müller *et al.*, 2004; Agarwal *et al.*, 2013). Taken together, the cAMP/PKA pathway plays an important role in morphological development and pathogenesis in non-pathogenic and pathogenic fungi.

1.1.3 The cAMP/PKA pathway in *Aspergillus fumigatus*

The components of cAMP/PKA signaling pathway in *A. fumigatus* have been identified and characterized. The components are (1) the two G protein coupled receptors (GPCRs): GprC and GprD (Grice *et al.*, 2013); (2) the heterotrimeric G protein: GpaA (α), GpaB (α), GanA (α), SfaD (β) and GpgA(γ) (Liebmann *et al.*, 2003; Lafon *et al.*, 2006); (3) the adenylyl cyclase AcyA

(Liebmann *et al.*, 2003); and (4) the regulatory subunit and two catalytic subunits of PKA PkaR, PkaC1 and PkaC2 (Liebmann *et al.*, 2004). Deletion mutants of the GPCR (∆*gprC and* ∆*gprD*) resulted in impaired growth. They had delayed mortality and reduced virulence in the animal model of aspergillosis respectively (Gehrke *et al.*, 2010). In contrast to the GPCR deletion strains, the ∆*gpaB* mutant shows normal growth but had reduced conidiation, whereas the adenylyl cyclase deletion strain has not only decreased growth but also showed severely impaired sporulation (Liebmann *et al.*, 2003). Further studies in the regulatory and catalytic subunits of PKA revealed that loss of PkaR results in reduced growth and germination rate as well as in conidiation. The ∆*pkaC1* mutant has similar phenotypes as the ∆*pkaR* mutant (Liebmann *et al.*, 2004; Zhao *et al.*, 2006; Grosse *et al.*, 2008; Fuller *et al.*, 2011). All of the studies above show that deletions of component being part of the cAMP/PKA pathway lead to attenuated virulence of the pathogenesis of *A. fumigatus*.

1.2 Adhesion

1.2.1 Adherence is required for fungal pathogenesis

To establish infections in the host, the spores of both plant and animal fungal pathogens should require the ability to adhere to the host cells. To bind to different surfaces, spores requires adhesins or hydrophobins which are proteins with adhesive or hydrophbic function. For instance, the CalA adhesin of the conidial cell surface in *A. fumigatus* promotes binding to laminin. Hydrophobin Hyd2 in *Beauveria bassian* and Hyd3 in *Clonostachs rosea* are important for adhesion of conidia to epicuticle in insects and root colonization in plants (Upadhyay *et al.*, 2009; Zhang *et al.*, 2011; Dubey *et al.*, 2014).

Hydrophobins are small amphiphilic proteins comprised of approximately 100 amino acids with eight conserved cysteine residues. They are less than 20 kDa in size, and are only found in filamentous fungi (Jensen *et al.*, 2010; Bayry *et al.*, 2012). The function of hydrophobin includes formation of aerial hyphae which

are essential for sporulation and adherence to hosts cells in pathogenic fungi (Linder *et al.*, 2005; Bayry *et al.*, 2012). For example, hydrophobin Mpg1 in plant pathogen *Magnaporthe grisea* is involved in conidiation and appressorium formation. Loss of this cell wall protein reduces virulence in rice (Talbot *et al.*, 1993; Talbot *et al.*, 1996).

Yeasts lack hydrophobins, instead they use adhesins which are cell wall proteins for adherence to different surfaces. Typical adhesins are a glycosyl phosphatidyl inositol (GPI) linked cell wall proteins and consist of three different domains (Verstrepen *et al.*, 2004) (Figure 2). The N-terminal domain plays an important role in binding of ligands and the amino acid sequences are highly conserved within the same family of adhesins. For instance, the N-terminal domain of four *FLO* (flocculins) genes in yeast have a PA14 and Flo5 domain (Brückner and Mösch, 2011). This ligand binding domain is followed by a serine and threonine rich region that contains many tandem repeats. The C-terminal region harbors a GPI anchor domain which is important for the attachment within the membrane (de Groot *et al.*, 2003). To date, more than 20 different adhesins have been identified in fungi and they are required for adherence to abiotic and biotic surfaces, biofilm formation and pathogenesis (Dranginis *et al.*, 2007; Linder and Gustafsson, 2008; de Groot *et al.*, 2013).

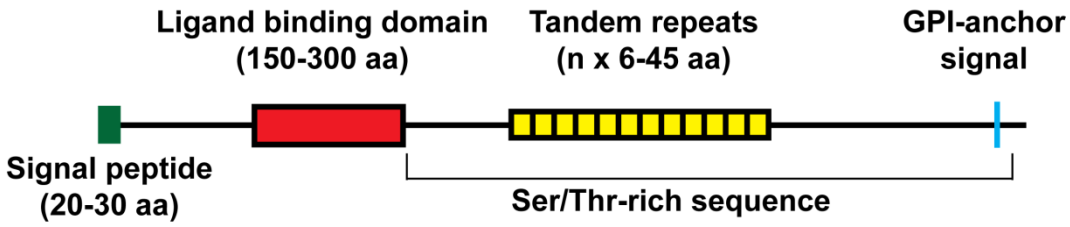


Figure 2. Typical structure of fungal adhesin.

The N-terminus contains the signal peptide and the ligand binding domain, which is required for adhesion. They are followed by the tandem repeats, which are rich in serine and threonine residues and are highly glycosylated. The C-terminus carries a signal for glycosyl phosphatidyl inositol (GPI) anchor (adapted from (Linder and Gustafsson, 2008)).

1.2.2 Adhesion in yeasts

The current understanding of adhesion and adhesins is obtained from studies in dimorphic yeasts as *S. cerevisiae* or *C. albicans*. The budding yeast can perform a morphological change from single vegetative cells into multi-cellular growth. This includes flocculation, biofilm formation and formation of filaments by activating the expression of specific cell wall associated adhesins that control cell-cell or cell-surface adhesion (Verstrepen and Klis, 2006; Brückner and Mösch, 2011) (Figure 3). *S. cerevisiae* contains five *FLO* genes encoding adhesins (*FLO1*, *FLO5*, *FLO9*, *FLO10* and *FLO11*) that contribute to the adhesive phenotypes. In the laboratory strain Σ1278b the expression of *FLO11* gene can be activated by nitrogen starvation while the other four *FLO* genes are transcriptionally silent (Dranginis *et al.*, 2007; Brückner and Mösch, 2011). In contrast, the other laboratory strain S288c *FLO1* and *FLO11* adhesins expression can only be activated when the transcription factor *ScFLO8* is reintroduced (Fichtner *et al.*, 2007) (Figure 3).

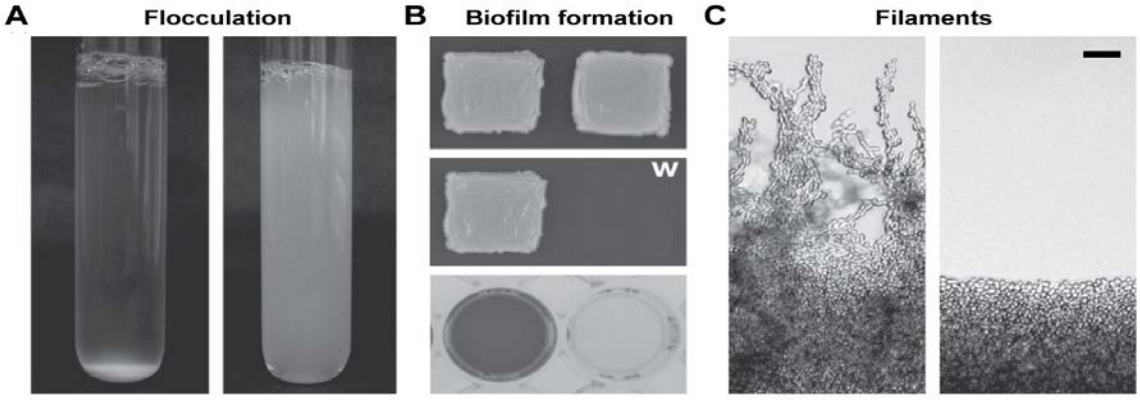


Figure 3. Adhesive phenotypes in *Saccharomyces cerevisiae*.

(A) Flocculation of a flocculent strain (left) and a non-flocculent strain (right). (B) Biofilm formation on solid (top) and in liquid media (bottom). On solid media, the adherent strain forms biofilm and can not be washed away (w), while the non-adherent strain (Δ*flo11*) can be removed by washing. In liquid media, the adherent strain forms a biofilm on plastic surfaces. (C) Filaments are observed on solid media the diploid *FLO11* expressing strain (left) is able to form filaments. No filaments are formed in a Δ*flo11* mutant (right) (modified from (Brückner and Mösch, 2011)).

The human pathogenic yeast *C. albicans* lives on mucosal surfaces such as oropharynx and vaginal tract (Southern *et al.*, 2008). The adherence to cells or abiotic surfaces including polystyrene and medical devices are mediated by various adhesins such as Als (agglutinin-like sequence), Hwp1 (hyphal wall protein) and Iff/Hyr (hyphally upregulated protein) protein families (Dranginis *et al.*, 2007; Hoyer *et al.*, 2008; de Groot *et al.*, 2013). These adhesins are highly expressed in hyphae growth and the transition from yeast form to hyphal form has been shown to be required for pathogenesis (Whiteway and Oberholzer, 2004). Similar to *S. cerevisiae*, the Flo8 transcription factor in *C. albicans* is essential for hyphal formation and the CaFlo8 can restore filamentous growth in a Δ*flo8* mutant in *S. cerevisiae* (Cao *et al.*, 2006).

Similar to *C. albicans*, the human pathogens *Candida glabrata* and *C. neoformans* regulate adhesion via its adhesins (de Groot *et al.*, 2013). Epa (epithelial adhesin) protein family containing the conserved PA14 (anthrax protective antigen) domain which is responsible for ligand binding (Rigden *et al.*, 2004). These adhesins in *C. glabrata* are required for adherence to epithelia cells during infection (Dranginis *et al.*, 2007; de Groot *et al.*, 2008). To date, only one protein, Cfl1, was reported as adhesin in *C. neoformans*. This protein regulates cell adhesion and biofilm formation, while overexpression of this gene shows reduced virulence (Wang *et al.*, 2012).

1.2.3 The transcription factor Flo8 is a regulator for adhesion

The ScFlo8 was first described as an essential gene for pseudohyphal growth in diploid and flocculation and invasive growth in haploid yeasts (Liu *et al.*, 1996). Further studies showed that this transcription factor is activated by Tpk2 which is one of the three catalytic subunit of PKA. Loss of either ScFlo8 or Tpk2 results in abolishment of pseudohyphal growth in *S. cerevisiae* (Pan and Heitman, 1999). The ScFlo8 is one of various activating and repressing regulators of *FLO11* expression which is essential for adhesive phenotype in budding yeast

(Rupp *et al.*, 1999; Braus *et al.*, 2003; Cullen and Sprague, 2012) (Figure 4). Also Flo8 in *C. albicans* can mediate adhesion and development (Cao *et al.*, 2006). Both ScFlo8 and CaFlo8 contain a conserved LUFS (LUG/LUH-Flo8-singal-stranded DNA binding) domain at the N-terminus. This indicate that ScFlo8 and CaFlo8 may share the same function. Further, CaFlo8 has been shown to be activated by CaTpk2 and regulates expression of virulence factors with another transcription factor Efg1 which belongs to APSES (Asm1, Phd1, Sok2, Efg1 and StuA) protein family (McDonough and Rodriguez, 2012) .

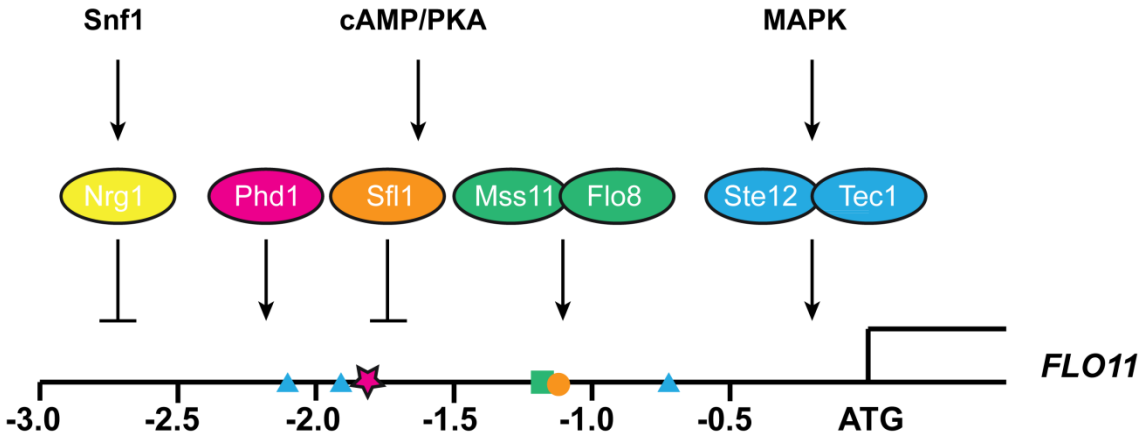


Figure 4. Expression of *FLO11* is regulated by various signaling pathways.

Flo11 is a major adhesin for adhesion in *S. cerevisiae* and its expression is controlled by different transcriptional activators or repressors. Mitogen activated protein kinase (MAPK) activates downstream targets (Ste12 and Tec1) to activate *FLO11* transcription. cAMP/PKA pathway regulates Flo8/Mss11, Sfl1 and Phd1 for expression of *FLO11*. Protein kinase Snf1 inactivates Nrg1 which is repressor of *FLO11* gene. Position of DNA binding sites for transcription factors on 3 kb *FLO11* promoter are colored (Adapted from (Octavio *et al.*, 2009)).

1.2.4 Adhesion in filamentous fungi

In comparison to yeast, little is known about adhesion in filamentous fungi. As a protein specifically expressed in filamentous fungi, hydrophobins are potential candidates for adhesion. Several studies have shown that these cell wall proteins is involved in adherence to hosts as well as development (Talbot *et al.*, 1996; Kim *et al.*, 2005; Linder *et al.*, 2005; Klimes and Dobinson, 2006;

Zhang *et al.*, 2011). Disruption of hydrophobins results in reduced virulence in pathogenic fungi (Talbot *et al.*, 1993; Sevim *et al.*, 2012). Recently, several adhesins have been identified to be responsible for adherence to different surfaces (Hung *et al.*, 2002; Wang and St Leger, 2007; Upadhyay *et al.*, 2009; Levdansky *et al.*, 2010; Wang *et al.*, 2012). In the entomopathogenic fungus *Metarhizium anisopliae*, the adhesin MAD1 is responsible for adherence to insect cuticle. Disruption of this gene results in reduced adherence and virulence in insects (Wang and St Leger, 2007). The Som1 protein, a Flo8 homolog in *M. oryzae*, is functional exchangeable with the yeast homologous protein. It can mediate adhesion by regulating hydrophobin Mpg1 expression and interacts with the APSES protein StuA. Deletion of *som1* gene causes loss of asexual and sexual development and impaired pathogenesis (Yan *et al.*, 2011).

1.2.5 Adhesion in *Aspergillus fumigatus*

Conidial adhesion to host epithelial cells in alveoli is an initial step in pathogenicity in *A. fumigatus* (Filler and Sheppard, 2006). Hyphal adhesion also plays a role in invasive infection because it is required for angiogenesis of hyphae fragment and dissemination in blood vessels (Abad *et al.*, 2010). It has been shown that cell wall proteins and carbohydrates play a role in adhesion (Figure 5).

Typically for filamentous fungi, *A. fumigatus* contains 4-6 hydrophobins (Beauvais *et al.*, 2007; Jensen *et al.*, 2010) and two of them (RodA and RodB) have been characterized in more detail (Parta *et al.*, 1994; Thau *et al.*, 1994; Paris *et al.*, 2003; Carrion Sde *et al.*, 2013). RodA is present on the surface of conidia and is responsible for conidial adherence to albumin and collagen but not laminin and fibrinogen. Δ*rodA* mutant results in normal virulence in animal model of invasive aspergillosis (Thau *et al.*, 1994; Sheppard, 2011). The Δ*rodB* mutant shows normal rodlet layer on conidia and a similar killing rate by alveolar macrophages in comparison to the wild type strain (Paris *et al.*, 2003). Apart from

hydrophobins, studies in *C. albicans* and *S .cerevisiae* show that adhesins are required for fungal adhesion.

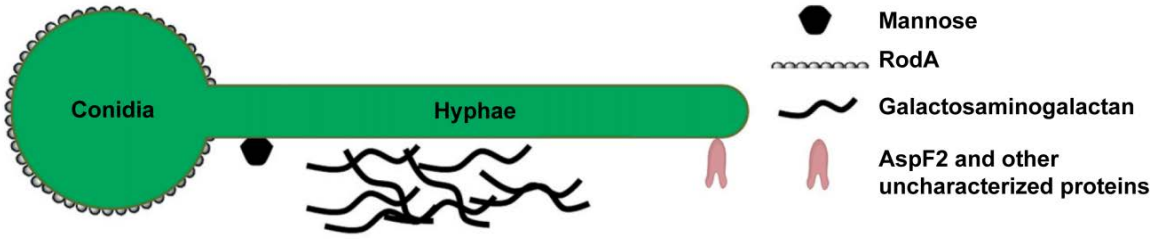


Figure 5. Adhesive molecule in *Aspergillus fumigatus*.

Hydrophobin (RodA) is required for conidial adherence. Galactosaminogalactan and mannose are cell wall carbohydrates for adhesion. *Aspergillus* allergen (AspF2) and other cell wall proteins are responsible for adherence to different molecules (modified from (Sheppard, 2011)).

By using bioinformatic tools, more than 100 proteins were predicted as putative adhesins in *A. fumigatus* (Upadhyay *et al.*, 2009; Chaudhuri *et al.*, 2011). One protein, CspA is involved in conidial adherence to extracellular matrix of A549 alveolar basal epithelial cells, but the deletion mutant showed no effect on virulence (Levdansky *et al.*, 2007).

More recently, several studies in cell wall carbohydrates, including chitin, galactomannan and galactossaminogalactan, indicate that these molecules play a role in adhesion. The UDP galactopyranose mutase converts UDP-galactopyranose to UDP-galactofuranose which links to galactomannan and glycoproteins in cell wall. Loss of this proteins results in increased conidial adherence in *A. fumigatus*, but shows normal virulence compared to the wild type (Lamarre *et al*., 2009). Gravelat *et al* (2013) identified another protein Uge3 which takes part in galactosaminogalactan formation and the disruption of *uge3* gene showed reduced adherence to plastic surfaces as well as fibronectin and A549 epithelial cells. Further, the Δ*uge3* mutant is avirulent in mice model of invasive aspergillosis (Gravelat *et al.*, 2013).

1.2.6 Asexual development

The asexual reproduction of *Aspergilli* has been well studied in the model fungus *Aspergillus nidulans*, and this cellular process can be divided into five steps while *A. fumigatus* has only four steps for asexual sporulation (Adams *et al.*, 1998; Yu, 2010). At the begin of asexual development, the vegetative hyphae form thick walled cells, the foot cells, at the interface of air and extend into air to produce aerial hyphae (stalk). A multinucleate vesicle is formed at the tip of aerial hyphae by swelling and followed by formation of metulae and phialides in *A. nidulans* or only phialides in *A. fumigatus* on the tip of vesicle using budding-like process. The first conidium is formed at the tip of phialides and long chains of uninucleate asexual conidiospores are formed by repeating conidium formation (Etxebeste *et al.*, 2010; Yu, 2010; Park and Yu, 2012) (Figure 6).

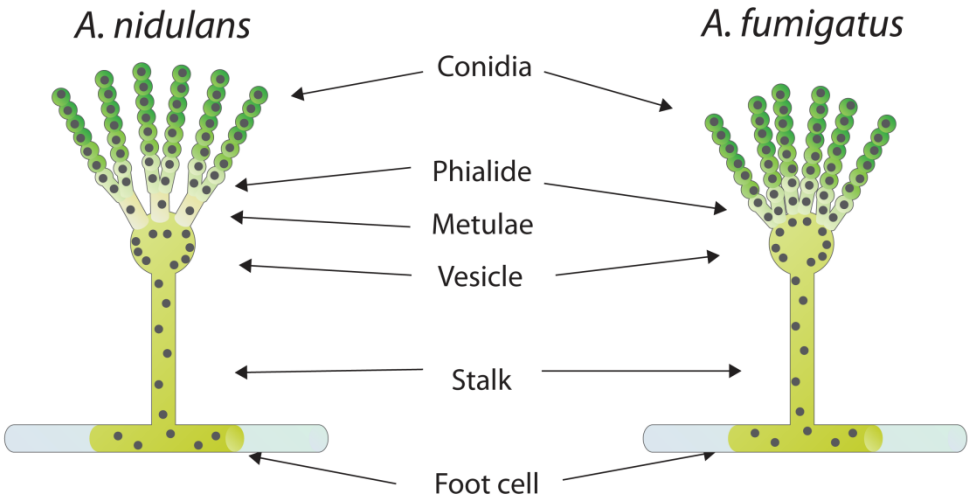


Figure 6. Asexual development of *Aspergillus nidulans* and *Aspergillus fumigatus*.

Conidiophores of *A. nidulans* and *A. fumigatus*. In contrast to *A. nidulans,* no metulae layers are formed in *A. fumigatus* (modified from (Yu, 2010)).

1.2.7 Regulation of asexual development

Conidiation in *Aspergilli* is a precise program which requires temporal and spatial control of various regulatory proteins. Three transcription factors named BrlA (*bristle*), AbaA (*abacus*) and WetA (*wet-white*) have been shown to play a central role in asexual development and they are conserved in *A. fumigatus* and *A. nidulans* (Adams *et al.*, 1998; Yu, 2010) (Figure 7). The BrlA is a C_2H_2 zinc finger transcription factor and is required for vesicle formation at the tip of aerial hyphae as well as the expression of *abaA* and *wetA* which are the downstream regulators. The Δ*brlA* mutant is impaired in conidiation and has longer stalks than wild type. The overexpression of *brlA* gene leads to conidiation from hyphae (Prade and Timberlake, 1993; Yu, 2010). The *abaA* gene encodes transcription factor containing a TEA (Tef-1 and Tec1, AbaA) DNA binding domain. The expression of *abaA* is activated by BrlA, and is required for differentiation of sterigmata which include metulae and phialides (Adams *et al.*, 1998). The Δ*abaA* mutant produces metulae at the tip of vesicle but not phialide (Boylan *et al.*, 1987; Tao and Yu, 2011). The last transcription factor WetA is required for conidiophores maturation and its expression is activated by AbaA. Loss of *wetA* gene results in normal conidiophores formation but not pigmentation on conidia (Marshall and Timberlake, 1991).

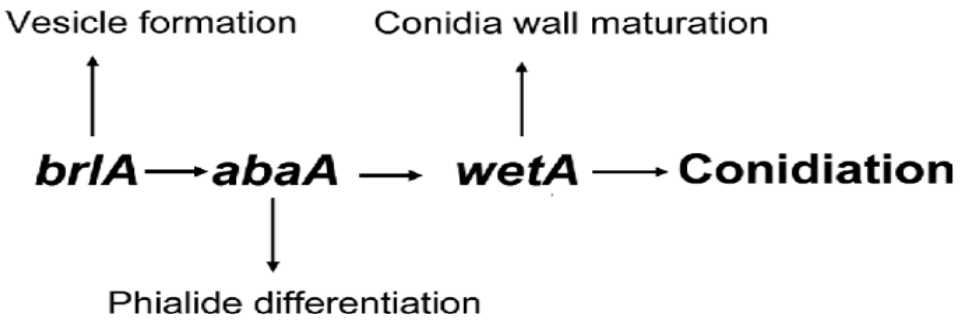


Figure 7. Central regulatory genes for asexual development in *Aspergillus fumigatus*.

Central regulation of conidiation. BrlA activates the expression of *abaA*, which activates transcription of downstream target *wetA*.

Besides the central regulatory proteins, other transcription factors can affect the expression of *brlA*, *abaA*, and *wetA* and are consequently required for normal asexual development. VosA is a transcription factor of the velvet family, which includes other velvet-domain proteins like VeA, VelB, and VelC, which is conserved in filamentous fungi (Bayram and Braus, 2012; Park *et al.*, 2012). Deletion of *vosA* shows constitutive conidiation and increased *brlA* expression, whereas its overexpression blocks asexual development (Ni and Yu, 2007; Park and Yu, 2012). MedA (*medusa*) and the APSES protein StuA (*stunted*) are developmental modifiers and are required for spatial distribution of BrlA and temporal expression of *brlA* gene, respectively (Adams *et al.*, 1998; Krijgsheld *et al.*, 2013). Deletion of *stuA* gene results in abnormal conidiation which has no metulae and phialides (Wu and Miller, 1997; Sheppard *et al.*, 2005). The Δ*medA* mutant in *A. nidulans* shows multiple layers of sterigmata, while the deletion of *medA* in *A. fumigatus* results in a similar phenotype as Δ*stuA* mutant showing the impaired formation of metulae and phialides (Sewall *et al.*, 1990; Gravelat *et al.*, 2010).

1.2.8 Upstream regulation of *brlA* expression

The heterotrimetric G protein and cAMP/PKA signaling pathway play a role in asexual development in *Aspergillus*. The Gα subunit FadA (*fluffy autolytic dominant*) of the G protein in *A. nidulans* negatively regulates asexual development. FadA activates the cAMP/PKA pathway and leads to vegetative growth and represses expression of the *brlA* gene (Yu *et al.*, 1996). GpaA, the FadA homolog in *A. fumigatus*, has been shown to share similar function to FadA and cause reduced conidiation in its dominant active form (Mah and Yu, 2006). But in contrast to FadA, the other Gα subunit GanB in *A. nidulans* which inhibits expression of *brlA* and asexual development (Chang *et al.*, 2004), the homolog in *A. fumigatus* promotes conidiation (Liebmann *et al.*, 2004). Recently, the Gβ-like protein, CpcB (cross pathway control B), has been shown to be required for proper expression of *brlA* in both *A. nidulans* and *A. fumigatus* (Kong *et al*., 2013).

Apart from the cAMP/PKA pathway, six upstream genes (*fluG*, *flbA*, *flbB*, *flbC*, *flbD*, and *flbE*) are also involved in the regulation of *brlA* transcription (Yu, 2010). Among these genes, the Δ*flbB*, Δ*flbC*, Δ*flbD* and Δ*flbE* mutants show the fluffy phenotype, which shows undifferentiated hyphae, and a low *brlA* expression in *A. nidulans* (Wieser *et al.*, 1994) (Figure 8). FlbB is a basic leucine zipper transcription factor (Etxebeste *et al*., 2008), and is usually localized at hyphal tips together with the transcription factor FlbE, which contains no conserved domain (Garzia *et al.*, 2009). Further studies show that the expression of *brlA* is lost in either Δ*flbB* or Δ*flbE* mutant. These results indicate that FlbB and FlbE interact with each other and that this complex is required for conidiation (Garzia *et al.*, 2009). In addition, FlbB has been shown to activate the expression of the *flbD* gene, which encodes a c-Myb transcription factor. FlbB is proposed to form a transcriptional complex with FlbD for regulation of *brlA* expression (Etxebeste *et al.*, 2010; Garzia *et al.*, 2010). In *A. fumigatus*, only FlbB and FlbE have been characterized. In contrast to *A. nidulans*, both deletion mutants show delayed *brlA* expression and reduced conidiation while the expression of *flbD* is absent in either Δ*flbB* or Δ*flbE* mutants (Kwon *et al.*, 2010b; Xiao *et al.*, 2010).

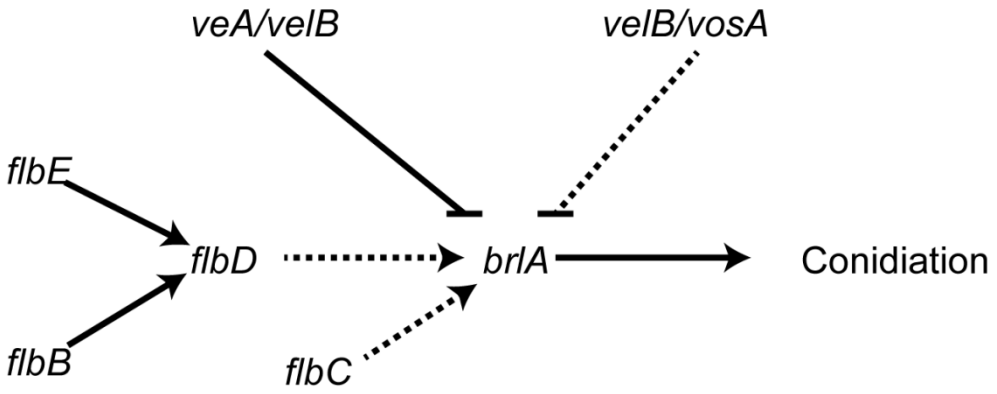


Figure 8. Model of regulation of *brlA* expression in *Aspergillus fumigatus*.

Upstream regulatory and velvet family genes regulate the *brlA* expression. Dashed lines indicate that this regulation has been shown in *Aspergillus nidulans* but not shown in *A. fumigatus* (modified from (Yu, 2010; Park *et al.*, 2012)).

1.3 Scope and aim of this study

Signaling pathway is required for communication between environments and organisms. This cross talk is important for pathogens to adapt to the environment which has limited nutrients within the hosts. The conserved cAMP/PKA signaling pathway plays a role in nutrient sensing and consequently regulates morphological development and virulence in fungi (Fuller and Rhodes, 2012). The downstream transcription factor Flo8 controls morphological transition and adhesion, which is required for pathogenesis, in dimorphic yeast *C. albicans* and *S. cerevisiae* (Brückner and Mösch, 2011; Mayer *et al.*, 2013). Previous studies showed that defects of cAMP/PKA pathway result in an attenuation of virulence in *A. fumigatus* (Gehrke *et al.*, 2010; Fuller *et al.*, 2011). This indicates that components of this pathway are potential targets for antifungal strategy. However, the knowledge of downstream targets in *A. fumigatus* is limited. This raised the question whether the *FLO8* homolog in *A. fumigatus* functions downstream of cAMP/PKA pathway and plays a role in development and adhesion.

The first part of this study was to test whether *FLO8/som1* homologs shared the similar function in *S. cerevisiae*. Flo11 adhesin is regulated by ScFlo8 and is required for adhesion in yeast (Fichtner *et al.*, 2007). The role of AfuSomA in adhesion and in activation of *ScFLO11* expression will be verified. A functional complementation study by expressing *AfusomA* in *S. cerevisiae* ∆*flo8* mutants and investigating the phenotypical changes in regard to adhesion and pseudohyphal growth. Further investigations on *FLO11* promoter binding were followed. The second part of this study was to characterize AfuSomA in *A. fumigatus*. The Som1 in *M. oryzae* regulates asexual/sexual development (Yan *et al.*, 2011). The function of AfuSomA was addressed by genetic studies. For this a deletion mutant and inducible down-regulation mutants were generated to test the ability of AfuSomA to regulate asexual development and adhesion. Transcription analysis will be performed to verify whether the regulatory genes in conidiation and adhesion are regulated by AfuSomA. Furthermore, the egg

model of invasive aspergillosis will be performed to test the role of AfuSomA in pathogenesis. The adhesins are the major component of adhesion in yeasts, and many putative adhesins were predicted by using bioinformatic tools (Upadhyay *et al.*, 2009; Chaudhuri *et al.*, 2011). Deletion strains of five putative adhesins were constructed and the phenotypical changes of these mutants will be characterized to address the question whether these proteins are required for adhesion. Flo8 regulates expression of target genes by complex Flo8-Mss11-Mfg1 in yeasts (Ryan *et al.*, 2012), and is activated by catalytic subunits of PKA (Pan and Heitman, 1999). The interaction partners of AfuSomA will be identified by a proteomic approach using GFP-Trap. This revealed the interesting question whether Flo8/Som1-Mss11-Mfg1 complex is conserved downstream of cAMP/PKA pathway.

2 Material and Methods

2.1 Materials

2.1.1 Growth media and growth conditions

Chemicals for media, buffer and solutions were purchased from AppliChem GmbH (Darmstadt, D), Carl Roth GmbH and Co. KG (Karlsruhe, D), Merck (Darmstadt, D), SERVA Electrophoresis GmbH (Heidelberg, D), Invitrogen GmbH (Karlsruhe, D), Roche Diagnostics GmbH (Mannheim, D), BD Becton Dickinson GmbH (Heidelberg, D), Novozyme (Bordeaux, F) and Sigma-Aldrich Chemie GmbH (Steinheim, D).

2.1.2 Media and conditions for *Escherichia coli*

Escherichia coli strains were grown in Lysogenic broth (LB) medium (0.5 % yeast extract, 1 % bacto-tryptone, 1 % NaCl) (Bertani, 1951) at 37 °C. 2 % agar was added for solid medium. For selection, 100 μg/ml ampicillin was used. Liquid cultures were grown on a shaker.

2.1.3 Media and conditions for *Saccharomyces cerevisiae*

Saccharomyces cerevisiae strains were cultivated at 30 °C in either non-selective YEPD medium (1 % yeast extract, 2 % peptone and 2 % glucose) or in SC-3 medium (0.15 % yeast nitrogen base without amino acid and $(NH_4)_2SO_4$, 0.5 % $(NH_4)_2SO_4$, 2 % glucose and 0.2 % amino acid mixture lacking uracil, L-methionine and L-leucine). The appropriate amino acids were supplemented as required. 2 % agar was added for solid medium. Liquid cultures were grown on a rotating platform.

2.1.4 Media and conditions for *Aspergillus fumigatus*

Aspergillus fumigatus strains were grown at 37 °C in minimal medium (MM) (10 g/l glucose, 1.84 g/l $(NH_4)_2$-tartrate, 0.52 g/l KCl, 1.52 g/l KH_2PO_4, 0.52 g/l $MgSO_4$, and 1 ml of the trace element solution [17.9 mM $FeSO_4$, 171.1 mM Na_2EDTA, 76.5 mM $ZnSO_4$, 177.9 mM H_3BO_3, 25.3 mM $MnCl_2$, 6.7 mM $CoCl_2$, 10.7 mM $CuSO_4$, and 0.9 mM $(NH_4)_6Mo_7O_{24}$ and pH 6.5], adjust pH to 6.5 with NaOH) (Käfer, 1977; Krappmann and Braus, 2005). 2 % agar was added for solid medium and 1 μg/ml pyrithiamine was used for selection of strains containing the *ptrA* resistance marker (Kubodera *et al.*, 2000). To remove the pyrithiamine resistance marker from the strains carrying the recyclable marker system (Hartmann *et al.*, 2010), MM was supplemented with 1 % xylose. For conditional expression experiments, either 1 % xylose or 5 mg/l doxycycline was added into MM. For egg infection experiment, strains were grown on malt extract agar (Oxoid, Basingstoke, UK).

2.2 Strains, plasmids and primers

2.2.1 *Escherichia coli* strain

E. coli DH5α (F^-, Φ80dΔ (lacZ) $M15^{-1}$, Δ (lacZYA-argF) U169, recA1, endA1, hsdR17 (rK^-, mK^+), supE44, λ^-, thi1, gyrA96, relA1) (Woodcock *et al.*, 1989) was used for general cloning.

2.2.2 *Saccharomyces cerevisiae* strains

S. cerevisiae strains BY4742 (*MATα*, *his3Δ1*; *leu2Δ0*; *lys2Δ0*; *ura3Δ0*), Y16870 (*MATα*, *his3Δ1*; *leu2Δ0*; *lys2Δ0*; *ura3Δ0*; *Δflo1*::*kanMX4*) (Euroscarf collection), RH2656 (*MATa/α*; *ura3-52/ura3-52*; *trp1*::*hisG/TRP1*) and RH2660 (*MATa/α*;*Δflo8*::*KanR/Δflo8*::*KanR*; *ura3-52/ura3-52*; *trp1*::*hisG/TRP1*) (Braus *et al.*, 2003) were used for heterologous expression of *A. fumigatus* protein. BY4742 and RH2660 were transformed with the plasmids pME2787, pME4194,

pME4195 or pME4197. RH2656 was transformed with pME2787 as control. BY16870 was transformed with the plasmids pME2786, pME4192, pME4193 or pME4196 and subsequently with plasmids containing the β-galactosidase reporter gene (Rupp *et al.*, 1999).

.

2.2.3 *Aspergillus fumigatus* strains

A. fumigatus strains which were used or generated in this study are listed in Table 1. Construction details are described below.

Table 1. *A. fumigatus* strains used in this study.

Strain	Genotype	Reference
A1176	*AfupyrG1*; Δ*AfubrlA*::*AfupyrG*$^{+}$	(Tao and Yu, 2011)
AfS35	Δ*akuA::loxP*	(Krappmann *et al.*, 2006)
AfGB72	Δ*akuA::loxP*; Δ*AfusomA*::*ptrA*	This study
AfGB73	Δ*akuA::loxP*; Δ*AfusomA*::*[AfusomA]*::*ptrA*	This study
AfGB74	Δ*akuA::loxP*; *ptrA*::^{p}*Tet*::*AfusomA*	This study
AfGB75	Δ*akuA::loxP*; *AfusomA::sgfp::ptrA*	This study
AfGB76	Δ*akuA::loxP*; ^{p}*gpdA::sgfp::his2a*^{t}, *ptrA*	I. Liewert, p.c.
AfGB77	Δ*akuA::loxP*; Δ*AfusomA::six*	This study
AfGB78	Δ*akuA::loxP*; *ptrA*::^{p}*xyl*::*AfusomA*	This study
AfGB79	Δ*akuA::loxP*; Δ*1g06480::six*	This study
AfGB80	Δ*akuA::loxP*; Δ*2g05150::six*	This study
AfGB81	Δ*akuA::loxP*; Δ*3g00880::six*	This study
AfGB82	Δ*akuA::loxP*; Δ*3g13110::six*	This study
AfGB83	Δ*akuA::loxP*; Δ*4g04070::six*	This study
AfGB84	Δ*akuA::loxP*; Δ*1g06480::six*; Δ*2g05150::six*	This study
AfGB85	Δ*akuA::loxP*; Δ*1g06480::six*; Δ*3g13110::six*	This study
AfGB86	Δ*akuA::loxP*; Δ*3g13110::six*; Δ*2g05150::six*	This study
AfGB87	Δ*akuA::loxP*; Δ*3g13110::six*; Δ*3g00880::six*	This study

Table 1. Continued.

Strain	Genotype	Reference
AfGB88	Δ*akuA::loxP*; Δ*3g13110::six*; Δ*4g04070::six*	This study
AfGB89	Δ*akuA::loxP*; Δ*4g04070::six*; Δ*2g05150::six*	This study
AfGB90	Δ*akuA::loxP*; Δ*3g13110::six*; Δ*3g00880::six*; Δ*4g04070::ptrA*	This study

t: terminator; p: promoter; p.c.: personal communication

2.2.3.1 Construction of *Aspergillus fumigatus* strains for *AfusomA* studies

To construct the *AfusomA* deletion strain, wild type (AfS35) was transformed with 3 μg of deletion fragment which was isolated from pME4188 using *Hin*dIII restriction enzyme. Transformants were selected with pyrithiamine to yield the Δ*AfusomA::ptrA* strain (AfGB72). Homologous integration was verified by Southern hybridization.

Complementation of the *AfusomA* deletion mutant was performed by transforming the complement fragment into the Δ*AfusomA::six* strain where the *ptrA* resistance marker was removed. The Δ*AfusomA::ptrA* mutant harbors the 5.3 kb recycle marker system containing a xylose driven β-recombinase, a *ptrA* resistance cassette and two flanking binding sites (*six*) for β-recombinase (Hartmann *et al.*, 2010). To generate the Δ*AfusomA::six* strain, the Δ*AfusomA::ptrA* mutant was streaked out on MM plates supplemented with 1 % xylose twice. Southern analysis was used to verify the Δ*AfusomA::six* mutant.

For complementation, the Δ*AfusomA::six* strain was transformed with 3 μg of complement fragment isolated from pME4190 by *Hin*dIII digestion to generate the complemented strain (AfGB73) with *ptrA* resistance marker. Homologous integration was verified by Southern hybridization.

To construct the conditional expression *AfusomA* strains, the xylose promoter from *Penicillium chrysogenum* (Hartmann *et al.*, 2010) and the Tet-On system (Helmschrott *et al.*, 2013) were used. 600 bp in front of the *AfusomA* gene was replaced by either the xylose promoter or the Tet-On system. The wild type was transformed either with the *Xyl-AfusomA* or the *Tet-AfusomA* which were excised from pME4199 or pME4191 using *Hin*dIII restriction enzyme. Transformants were selected with pyrithiamine to generate the *Xyl-AfusomA* strain (AfGB78) and the *Tet-AfusomA* mutant (AfGB74). Homologous integration was examined by Southern hybridization.

To generate the *AfusomA-sGFP* tagged mutant, the wild type strain was transformed with 3 μg of the sGFP fused fragment isolated from pME4198 using *Hin*dIII restriction enzyme and selected by pyrithiamine to yield the *AfusomA-sGFP* mutant strain. Homologous integration was verified by Southern hybridization.

2.2.3.2 Construction of single or multiple putative adhesin deletion mutants in *Aspergillus fumigatus*

In order to generate multiple adhesins deletion strains, the recyclable marker system was used to construct the deletion fragments. To construct the single deletion strain of putative adhesins, the wild type strain was transformed with different deletion fragments and the transformants were selected by pyrithiamine. The Δ*1g06480::ptrA* mutant was generated by transforming the deletion fragment isolated from pME4200 using *Hin*dIII restriction enzyme into wild type (AfS35). Further, this deletion mutant was streaked out on MM plates supplemented with 1 % xylose twice to yield the Δ*1g06480::six* strain (AfGB79) for multiple deletion. Southern analysis with 5' flanking region of *1g06480* as probe was used to examine homologous integration and marker recycle.

To construct the Δ*2g05150::ptrA* mutant, the wild type strain was transformed with the deletion fragment which was excised from pME4201 by

*Hin*dIII digestion. The *ptrA* resistance cassette was removed by streaking the Δ*2g05150::ptrA* mutant on xylose containing MM plates to generate the Δ*2g05150::six* mutant (AfGB80). The same procedure was applied to construct the Δ*3g00880*, Δ*3g13110* and Δ*4g04070* mutant. The deletion fragment of *3g00880* or *4g04070* was isolated from pME4202 or pME4204 using *Hin*dIII restriction enzyme, respectively. The deletion fragment of *3g13110* was amplified from pME4203 with the primers HO234/235. Homologous integration was verified by Southern hybridization.

To generate double adhesin deletion mutants, those single deletion strains where the *ptrA* resistance marker was removed were transformed with the corresponding deletion fragments. The Δ*1g06480::six* mutant was transformed with the deletion fragment from pCL3 and pME4203 to generate Δ*1g06480::*Δ*2g05150::ptrA* and Δ*1g06480::*Δ*3g13110*::ptrA double deletion strains. Similarly, the Δ*3g13110::six* mutant was used to construct three double adhesion deletion mutants including Δ*3g13110::*Δ*2g05150::ptrA*, Δ*3g13110::*Δ*3g00880::ptrA* and Δ*3g13110::*Δ*4g04070::ptrA* by transforming the corresponding deletion fragments excised from pME4201, pME4202 and pME4204, respectively. The Δ*4g04070::*Δ*2g05150::ptrA* double deletion mutant was generated by transforming the deletion fragment isolated from pME4201 into the Δ*4g04070::six* strain. In the double deletion the *ptrA* resistance marker mutants was removed as previously described and resulted in Δ*1g06480::*Δ*2g05150::six* (AfGB84), Δ*1g06480::*Δ*3g13110*::*six* (AfGB85), Δ*3g13110::*Δ*2g05150::six* (AfGB86), Δ*3g13110::*Δ*3g00880::six* (AfGB87), Δ*3g13110::*Δ*4g04070::six* (AfGB88) and Δ*4g04070::*Δ*2g05150::six* (AfGB89). The triple adhesion deletion strain was constructed by transforming the deletion fragment from pME4204 into the Δ*3g13110::*Δ*3g00880::six* strain to yield the Δ*3g13110::*Δ*3g00880::*Δ*4g04070*::*ptrA* mutant (AfGB90). Homologous integration was examined by Southern hybridization as previously described.

2.2.4 Plasmids

All plasmids used or constructed in this study are stated in Table 2. Construction details are given below.

Table 2. Plasmids used in this study.

Plasmid	Description	Reference
A. fumigatus		
pJET1.2	Cloning vector	Fermentas GmbH (St. Leon-Rot, D)
pUC19	Cloning vector	Fermentas GmbH (St. Leon-Rot, D)
pCH008	Plasmid contains Tet-On system and *ptrA* marker	(Helmschrott *et al.*, 2013)
pSK485	Plasmid contains recyclable marker driven by xylose promoter	(Hartmann *et al.*, 2010)
pME4188	5'flanking^{AfusomA}*::*recyclable marker*::*3'flanking^{AfusomA} in pJET1.2	This study
pME4189	4.6 kb of 5'flanking*::AfusomA* in pUC19	This study
pME4190	5'flanking:: *AfusomA*::recycable marker::3'flanking in pUC19	This study
pME4191	5'flanking^{AfusomA}::*ptrA*::*Tet-On*:: *AfusomA* in pUC19	This study
pME4198	5'flanking*::*AfusomA*::sgfp::*recyclable marker*::*3'flanking in pUC19	This study
pME4199	5'flanking^{AfusomA}::*ptrA*::^{p}*xyl*::*AfusomA* in pUC19	This study
pME4200	5'flanking^{1g06480}*::*recyclable marker*::*3'flanking^{1g06480} in pUC19	This study
pME4201	5'flanking^{2g05150}*::*recyclable marker*::*3'flanking^{2g05150} in pJET1.2	This study
pME4202	5'flanking^{3g00880}*::*recyclable marker*::*3'flanking^{3g00880} in pUC19	This study
pME4203	5'flanking^{3g13110}*::*recyclable marker*::*3'flanking^{3g13110} in pJET1.2	This study
pME4204	5'flanking^{4g04070}*::*recyclable marker*::*3'flanking^{4g04070} in pUC19	This study

Table 2. Continued.

Plasmid	Description	Reference
S. cerevisiae		
pME2786	pRS425 containing *MET25* promoter *CYC1* terminator, *LEU2*, *2μm*, *Amp*, *ori*	(Mumberg *et al.*, 1994)
pME2787	pRS426 containing *MET25* promoter *CYC1* terminator, *URA3*, *2μm*, *Amp*, *ori*	(Mumberg *et al.*, 1994)
pME2167	3 kb *ScFLO11* promoter in YEp355	(Rupp *et al.*, 1999)
p*FLO11-2/1* to p*FLO11-15/14*	400 bp *ScFLO11* promoter sequence fragments cloned into pLG669Z	(Rupp *et al.*, 1999)
pME4192	*AfusomA* cDNA in pME2786	This study
pME4193	*AfusomA* cDNA variant in pME2786	This study
pME4194	*AfusomA* cDNA in pME2787	This study
pME4195	*AfusomA* cDNA variant in pME2787	This study
pME4196	*ScFLO8* in pME2786	This study
pME4197	*ScFLO8* in pME2787	This study

t: terminator; p: promoter

2.2.4.1 Construction of plasmids for deletion and complementation of *AfusomA*

The 5' and 3' flanking regions (1 kb) of the *AfusomA* gene were amplified with the corresponding primers HO499/500 or HO501/502 to construct the deletion fragment. These two products were fused by amplifying with the primer pair HO499/502 to yield a 2 kb fragment which contains a restriction site for *Sfi*I in the middle and restriction site for *Hin*dIII at both ends. Then it was cloned into pJET1.2 Blunt cloning vector (Fermantas GmbH, St. Leon-Rot, D). The self excising marker system, which harbors a xylose driven β-recombinase, a pyrithiamine resistance cassette and two flanking binding sites (*six*), was isolated from pSK485 (Hartmann *et al.*, 2010) with *Sfi*I restriction enzyme. This 5.3 kb recyclable marker fragment was cloned into the corresponding restriction sites in the previous plasmid containing fused 5' and 3' flanking regions to generate pME4188 (Table 2).

For complementation, the 4.6 kb fragment, which harbors the *AfusomA* gene and the 2 kb upstream region was amplified with the primer pair HO603/601 and cloned into *Sma*I digested pUC19 (Fermantas GmbH, St. Leon-Rot, D) using the In-fusion HD Cloning Kit (Takara BioEurope/Clontech, Saint-Germain-en-Laye, F) to generate pME4189. Linear pME4189 was generated by amplifying with primers HO711/611. Using In-fusion Kit ,this linear plasmid was fused with the recyclable marker fragment isolated from pSK485 and the 3' flanking fragment of *AfusomA* which was amplified with the primer pair HO677/501 to yield pME4190.

2.2.4.2 Constructs for two conditional expression *AfusomA* and the *AfusomA::sgfp* plasmids

To construct the conditional expression *AfusomA* gene, the xylose promoter and the Tet-On system were chosen. To generate xylose dependent expression of *AfusomA*, the xylose promoter (1.7 kb) and the *ptrA* resistance cassette (2 kb) were amplified with the corresponding primers HO608/609 and HO115/116 using pSK485 (Hartmann *et al.*, 2010) as template. These two fragments were fused by amplifying with the primer pair HO115/609 to generate the *ptrA-xyl* promoter fused fragment (3.7 kb). This fragment replaced the 602 bp fragment in front of the *AfusomA* gene (position -602~-1) by cloning into linear pME4189 which was amplified with primers HO602/610 to yield pME4199.

Similar procedure was used to generate the Tet-On system regulated *AfusomA* gene. The fragment (4.1 kb) containing the *prtA* resistance cassette and the Tet-On system was amplified with the primer pair HO116/675 using pCH008 (Helmschrott *et al.*, 2013) as template. The Tet-On system fragment also replaced 602 bp fragment in front of the *AfusomA* gene (position -602~-1). This fragment was cloned into the linear pME4189 amplified with primers HO710/676 and resulted in the plasmid pME4191.

To construct C-terminus sGFP tagged AfuSomA, the sGFP gene was amplified with primers HO210/713 from pHI_4 (Table 2). The 3' flanking region of the *AfusomA* gene was amplified with the primer set HO648/677. The sGFP and 3' flanking region were cloned into linear pME4189 amplified with primers HO611/712 using In-fusion Kit. This temporary plasmid contained *AfusomA*-sGFP used gene together with 5' and 3' flanking regions. Then, the recyclable marker fragment isolated from pSK485 was cloned into the previous plasmid which was amplified with the primer HO697/501 to yield pME4198.

2.2.4.3 Construction of plasmids for adhesins deletion

To characterize five putative adhesins in *A. fumigatus*, plasmids containing deletion fragments were constructed. The 5' and 3' flanking regions (1 kb) of the *1g06480* gene were amplified with the corresponding primers HO555/556 or HO557/558 to construct the deletion fragment. The flanking regions of *1g06480* and the recyclable marker fragment isolated from pSK485 using *Sfi*I restriction enzyme were cloned into *Sma*I digested pUC19 plasmid using In-fusion Kit to yield pME4200.

To construct the *2g05150* deletion fragment, the 5' and 3' flanking regions (1 kb) were amplified with the corresponding primers HO503/504 and HO505/506. Two fragments were fused by amplifying with the primer pair HO503/506 resulting in 2 kb fragment. This fragment contained a restriction site for *Sfi*I in the middle and a restriction site for *Hin*dIII at both ends and was cloned into pJET1.2 plasmid. Further, the recyclable marker fragment isolated from pSK485 was cloned into the previous plasmid which was digested with *Sfi*I restriction enzyme to generate pME4201.

To generate the *3g00880* deletion fragment, the 3.8 kb fragment containing *3g00880* and its 5' and 3' flanking regions was amplified with the primer set HO654/657. This fragment was cloned into *Sma*I digested pUC19 using In-fusion Kit. The recyclable marker fragment was cloned into the previous

plasmid which was amplified with primers HO655/656 using In-fusion Kit to generate pME4202.

To construct the *3g13110* deletion fragment, the 5' and 3' flanking regions (1 kb) were amplified with the corresponding primers HO234/482 and HO235/483. Two fragments were fused by amplifying with the primer pair HO234/235 resulting in a 2 kb fragment which contains a restriction site for *Sfi*I in the middle. This fragment was cloned into pJET1.2 plasmid. Further, the recyclable marker fragment isolated from pSK485 was cloned into the previous plasmid which was digested with *Sfi*I restriction enzyme to generate pME4203.

The 5' and 3' flanking regions (1 kb) of the *4g04070* gene were amplified with the corresponding primers HO559/560 or HO561/562 to construct the deletion fragment. The flanking regions of *4g04070* and the recyclable marker fragment isolated from pSK485 using *Sfi*I restriction enzyme were cloned into *Sma*I digested pUC19 plasmid using In-fusion Kit to yield pME4204.

2.2.4.4 Construction of heterogeneous expression plasmids

To complement *flo8*-deficient yeast, two *AfusomA* cDNA variants were amplified from the cDNA pool with primer HO441/442 introducing *Spe*I and *Hin*dIII restriction sites on 5' and 3' ends, respectively. These two AfuSomA cDNA fragments were digested by *Spe*I and *Hin*dIII and cloned into corresponding digested pME2786 and pME2787 under *MET25* promoter resulting in pME4192, pME4193, pME4194 and p4195. For positive control, *S. cerevisiae* Flo8 (ScFlo8) was amplified with primer HO446/447 and cloned into *Sma*I digested pME2786 and pME2787 resulting in pME4196 and pME4197.

2.2.5 Primers

The primers that were used for plasmid construction and quantitative real-time PCR (qRT-PCR) are listed in Table 3. All primers were acquired from Eurofins MWG Operon (Ebersberg, D).

Table 3. Primers used in this study.

Primer	Sequence (5'→3')	Description
HO1	CTCTTCGAAGGCTGGACTTGC	*H2A* qPCR Forward
HO2	GGAGATGGCGAGGAATGATACG	*H2A* qPCR Reverse
HO41	TTGAGATGCGAAAGGATGTGGT	*vosA* qPCR Forward
HO42	GGCAAATGACCGAGAAAGGAAC	*vosA* qPCR Reverse
HO43	TGTAACTTCACTCCCGCCTCTG	*veA* qPCR Forward
HO44	TGATTCGTTTCCCACAATAGACG	*veA* qPCR Reverse
HO45	CTCCTACTTATCCCGCCTTCACA	*velB* qPCR Forward
HO46	GGCATCTGACCTCCAGCGTAAT	*velB* qPCR Reverse
HO47	CTCACCAGGGGGTCTCAAATG	*velC* qPCR Forward
HO48	CGGGGGTAGGGCTTGTATCA	*velC* qPCR Reverse
HO115	AATTGATTACGGGATCCCATTGGTAACG	*ptrA* Forward
HO116	TCTTGCATCTTTGTTTGTATTATACTGTC	*ptrA* Reverse
HO141	TCTATGCTCCACATCCCACCAA	*laeA* qPCR Forward
HO142	AAAGTCGCAATTTCTCGGGTGA	*laeA* qPCR Reverse
HO157	AAGCCTCATGTCTGCTGGGTTC	*brlA* qPCR Forward
HO158	CCGATAGTCCGGGTTGTAGTCG	*brlA* qPCR Reverse
HO210	CTACTTGTACAGTTCGTCCAT	sGFP Reverse
HO234	AAGAATCAAGCCCTCCAGGAGTCA	*3g13110* 5'flanking
HO235	CCGTCGTCGGGACATCATCTGT	*3g13110* 3'flanking
HO277	CCTGCCGTAACATTGCTTCTTG	*3g13110* qPCR Forward
HO278	CACAGTCATCATCCTCCGATCC	*3g13110* qPCR Reverse
HO441	ACTAGTATGAATCAGATGAATGTGACGGGG	*AfusomA* with *Spe*I site
HO442	AAGCTTCTGAAGAACCGACGGACTCATTTA	*AfusomA* with *Hin*dIII site
HO446	GGATCCATGAGTTATAAAGTGAATAGTTCGTATCC	*ScFLO8* with *Bam*HI site
HO447	CTCGAGGACTTCAGCCTTCCCAATTAATAAA	*ScFLO8* with *Xho*I site

Table 3. Continued.

Primer	Sequence (5'→3')	Description
HO482	CGCGTAATGGCCTGAGTGGCCGAGTTT GTATTTGTGTTAGAGCGACAG	*3g13110* 5'flanking with *Sfi*I site
HO483	ATTACGCGGCCATCTAGGCCTGTGCTTT GTTCCGTCCCAACA	*3g13110* 3'flanking with *Sfi*I site
HO499	AAGCTTCCGAACAAGCGATTTACGCC	*AfusomA* 5'flanking with *Hin*dIII site
HO500	GACCTATAGGCCTGAGGGTGGCACTGC GAGGAGTTT	*AfusomA* 5'flanking with *Sfi*I site
HO501	CATAATATGGCCATCTAATGAGTCCGTC GGTTCTTCAGTT	*AfusomA* 3'flanking with *Sfi*I site
HO502	AAGCTTAAGATAGGCTGTCAGGATTGTA CGG	*AfusomA* 3'flanking with *Hin*dIII site
HO503	AAGCTTCCAGGTTACACTGCCATTGGG	*2g05150* 5'flanking with *Hin*dIII site
HO504	GACCTATAGGCCTGAGTGGAGCAACCAA ACGGTATCAG	*2g05150* 5'flanking with *Sfi*I site
HO505	CATAATATGGCCATCTATGATCTGTCCG AGCATGGAATG	*2g05150* 3'flanking with *Sfi*I site
HO506	AAGCTTGGACGCCTGGATGGTCTTTCA	*2g05150* 3'flanking with *Hin*dIII site
HO555	TCGAGCTCGGTACCCAAGCTTATCTAGG CGATTCGCCTAA	*1g06480* 5'flanking with pUC19 overhang
HO556	TTGACCTATAGGCCTTTGAAAGATCGAC GACAGCG	*1g06480* 5'flanking with *six* overhang
HO557	AGCATAATATGGCCATGTTAGATGGAGT TAGGAGCCGG	*1g06480* 3'flanking with *six* overhang
HO558	CTCTAGAGGATCCCCTGGGTCTCTGCTC GGCTTAT	*1g06480* 3'flanking with pUC19 overhang
HO559	TCGAGCTCGGTACCCAAGCTTTCCGAGC AATGTCATCTGT	*4g04070* 5'flanking with pUC19 overhang
HO560	TTGACCTATAGGCCTGCTAGAGTTCGTA GTGCCGCA	*4g04070* 5'flanking with *six* overhang
HO561	AGCATAATATGGCCACCTTGTACTCCTC CCAGGAGAG	*4g04070* 3'flanking with *six* overhang
HO562	CTCTAGAGGATCCCCTACTTGCCTCGGC TATCACA	*4g04070* 3'flanking with pUC19 overhang
HO601	CTCTAGAGGATCCCCTGAAGAACCGACG GACTCATTTA	*AfusomA* with pUC19 overhanging

Table 3. Continued.

Primer	Sequence (5'→3')	Description
HO602	TCCCGTAATCAATTGTTCGATGGGCGACACGAA	*AfusomA 5'*flanking with *ptrA* overhang
HO603	TCGAGCTCGGTACCCAAGCTTCCTTCAAAGATAACCCCTA	*AfusomA* 5'flanking with pUC19 overhang
HO608	AACAAAGATGCAAGATAATAGTTAACTGCAGGCGGCC	xylose promoter with *ptrA* overhang
HO609	GTTGGTTCTTCGAGTCGATGAATG	xylose promoter Reverse
HO610	ACTCGAAGAACCAACATGAATCAGATGAATGTGACGGG	*AfusomA* with xylose promoter overhang
HO611	GGGGATCCTCTAGAGTTAACCTGCAG	pUC19 Forward
HO617	AACCGGTAATGCCCAGACAGAT	*AfusomA* qPCR Forward
HO618	GTGTCCGTTCATGTCCATGTCA	*AfusomA* qPCR Reverse
HO648	GAACTGTACAAGTAGAATGAGTCCGTCGGTTCTTCAGTT	*AfusomA* 3'flanking with sGFP overhang
HO654	TCGAGCTCGGTACCCGGGTCTGAGGGCTTCGATTCT	*3g00880* 5'flanking with pUC19 overhang
HO655	TTGACCTATAGGCCTGTGATAGCGAGTGATCGAATGTA	*3g00880* 5'flanking with *six* overhang
HO656	AGCATAATATGGCCAGCGTGAACCTTTTGTCATCTCAG	*3g00880* 3'flanking with *six* overhang
HO657	CTCTAGAGGATCCCCGGGCCGCCAGACCATCAT	*3g00880* 3'flanking with pUC19 overhang
HO660	GCTCTGACTCTCACTGCCTTCG	*3g00880* qPCR Forward
HO661	AAGCTTGTTGACGGGAGGGTAG	*3g00880* qPCR Reverse
HO675	GGTGATGTCTGCTCAAGCGG	Tet-On Reverse
HO676	GCTTGAGCAGACATCACCATGAATCAGATGAATGTGACGGG	*AfusomA* with Tet-On overhang
HO677	CTCTAGAGGATCCCCAAGATAGGCTGTCAGGATTGTACGG	*AfusomA* 3'flanking with pUC19 overhang
HO680	GTACCCATCAAAAGCCGTCCTC	*medA* qPCR Forward
HO681	TTCTGCATGCGAGTGAATTGAA	*medA* qPCR Reverse
HO682	CTCCTGAGCACGAGTCGGAATA	*stuA* qPCR Forward
HO683	CGTGGAGTCATACGTCCAGACC	*stuA* qPCR Reverse

Table 3. Continued.

Primer	Sequence (5'→3')	Description
HO684	CTCCAGAGCAAGCCTATCCACA	*flbB* qPCR Forward
HO685	TGCGGTACAGTTCGTGGTTCTT	*flbB* qPCR Reverse
HO686	GTGACGTTGAAGGGTGTGGAAG	*flbC* qPCR Forward
HO687	ACTCCTCCTCGCCACCAGATAC	*flbC* qPCR Reverse
HO688	AACCTGAAGCCCTCGTTGAATC	*flbD* qPCR Forward
HO689	TGGCCGAGAGACCTCTTCTCTT	*flbD* qPCR Reverse
HO697	TTGACCTATAGGCCTCTACTTGTACAGTT CGTCCAT	sGFP with *six* overhang
HO710	AACAAAGATGCAAGATTCGATGGGCGAC ACGAA	*AfusomA* 5'flanking with *ptrA* overhang
HO711	TTGACCTATAGGCCTTTATAAGCCATCTC CGGCGC	*AfusomA* with *six* overhang
HO712	TCCTCCTGATCCTCCTAAGCCATCTCCG GCGCC	*AfusomA* witih linker
HO713	GGAGGATCAGGAGGAATGGTGAGCAAG GGCGAGGAGCTG	sGFP with linker Forward
HO788	CCTATGGCCGTACCAAATGGAT	*uge3* qPCR Forward
HO789	GTGGGAGTCTGTCTGGGGTCTT	*uge3* qPCR Reverse

2.2.6 Sequencing

To sequence the plasmid, 300 ng plasmid DNA and 5 pmol primer were applied in a total volume of 5 µl. Sequencing was performed by the Göttingen Genomics Laboratory.

2.3 Molecular methods

2.3.1 Computational analysis

Blast searches and protein conserved domain identification were conducted at the National center for Biotechnology Information (www.ncbi.hlm.hin.gov). Protein alignments and phylogenetic trees were made by Clustal Omega and ClustalW2 at European Molecular Biology Laboratory – European Bioinformatics Institute (www.ebi.ac.uk). Nuclear localization signal (NLS) was predicted at cNLS mapper (http://nls-mapper.iab.keio.ac.jp/). The

protein name and gene number of *A. fumigatus* are according to the AspGD (http://www.aspergillusgenomes.org) (Arnaud *et al.*, 2012). The yeast homolog is according to the SGD (http://www.yeastgenome.org/) (Cherry *et al.*, 2012). Protein and DNA sequence analysis was performed using Lasergene software (Dna Star Inc., Madison, WI, USA).

2.3.2 Recombinant DNA method

Recombinant DNA technologies were performed according to the standard methods (Sambrook *et al.*, 1989). DNA fragments for plasmid construction, hybridization probes or sequencing were amplified by Polymerase Chain Reaction (PCR) with the *Taq*-(Fermentas GmbH/Thermo Fisher Scientific GmbH, St. Leon-Rot/Schwerte, D) or Phusion High-Fidelity (Finzymes/Thermo Fisher Scientific GmbH, Vantaa, FIN/Schwerte, D) polymerase. The PCR products were analyzed according to their size by agarose gel electrophoresis. Extraction of desired DNA fragment was performed using QIAquick Gel Extraction Kit (Qiagen GmbH, Hilden, D) following the user's manual. Restriction enzymes and T4 ligase were obtained from Fermentas GmbH/Thermo Fisher Scientific GmbH (St. Leon-Rot/Schwerte, D). DNA was eluted in 30 µl H_2O and stored at -20 °C. The DNA concentration was measured with the Nanodrop ND-1000 (Peqlab Biotechnologie GmbH, Erlangen, D).

2.3.3 Isolation of nucleic acids

2.3.3.1 Isolation of plasmid DNA from *Escherichia coli*

The *E. coli* strains were grown in 5 ml LB medium with 100 µg/ml ampicillin for 18 h at 37 °C for plasmid isolation. The purification of plasmid DNA was performed with the QIAprep Spin Miniprep Kit (Qiagen GmbH, Hilden, D) using the manufacturer's instructions. The plasmid was eluted in 50 µl H_2O and the concentration was determined by the Nanodrop ND-1000 (Peqlab Biotechnologie GmbH, Erlangen, D). Plasmid DNA was stored at -20 °C.

2.3.3.2 Isolation of genomic DNA from *Aspergillus fumigatus*

Isolation of genomic DNA from *A. fumigatus* was performed as previously described (Lee and Taylor, 1990). About 10^6-10^7 conidia were inoculated in 200 ml MM medium and incubated overnight at 37 °C on a shaker (180rpm). The mycelia were filtered with sterile miracloth (Merck KGaA, Darmstadt, D) and ground with a mortar in liquid nitrogen. 500 µl lysis buffer (50 mM Tris pH 7.5, 50 mM EDTA pH 8.0, 3% SDS, 1 % β-Mercaptoethanol) was added to 500 µl ground mycelia in 2 ml tube and the samples were vortexed for 10 s. Further, 500 µl phenol:chloroform solution (1:1) was added to the samples and vortexed for 5 min at 4 °C. After centrifugation for 15 min at 13000 rpm the supernatant was transferred to new 1.5 ml tube and mixed with 800 µl chloroform and vortexed. After another centrifugation for 15 min at 13000 rpm, the upper layer was transferred to new a 1.5 ml tube and mixed with 500 µl isopropanol. Afterwards, the samples were centrifuged for 5 min at 13000 rpm and the pellets were washed with 500 µl 70% ethanol. The samples were centrifuged for 2 min at 13000 rpm, then the pellets were dried for 25 min at 50 °C. Finally, the DNA was resuspended in 100 µl H_2O, mixed with 2 µl RNaseA solution (10mg/ml RNaseA, 10 mM Tris pH 7.5, 15 mM NaCl) and incubated for 45-60 min at 37 °C. Genomic DNA was stored at 4 °C. The concentration was determined by the Nanodrop ND-1000 (Peqlab Biotechnologie GmbH, Erlangen, D).

2.3.3.3 Isolation of RNA from *Aspergillus fumigatus*

The wild type, Δ*AfusomA* and *AfusomA* complemented strains were grown in 200 ml MM medium on a shaker (180 rpm) for 20 h at 37 °C. The harvested mycelium was ground in liquid nitrogen. Two spatulas of mycelia powder were mixed with 600 µl RLT buffer without β-mercaptoethanol. RNA isolation was performed using the RNeasy Plant Mini Kit (Qiagen GmbH, Hilden, D) referring to user's manual. RNA was eluted in 40 µl H_2O and stored at -20 °C. The

concentration was determined with the Nanodrop ND-1000 (Peqlab Biotechnologie GmbH, Erlangen, D).

2.3.4 Quantitative real-time PCR (qRT-PCR)

To perform qRT-PCR, the cDNA was synthesized from total RNA of *A. fumigatus* using the QuantiTect Reverse Transcription Kit (Qiagen GmbH, Hilden, D) following manufacturer's instructions. 800 ng RNA was used for cDNA synthesis and cDNA was stored at -20 °C. The qRT-PCR was performed using RealMaster SYBR Rox Kit (5 Prime GmbH, Hilden, D), 10 pmol of each primer and 1 µl 1:10 diluted cDNA in a total volume of 20 µl. The PCR was performed by the LightCycler 2.0 (Roche Diagnostics GmbH, Mannheim) using a twp step program with 33 cycles and 63 °C as annealing temperature. Each sample was performed in duplicates and the experiment was repeated three times. The histone H2A (3G05360) was used as endogenous reference. The relative expression of the gene of interest was calculated using the ΔCT method as previous described (Livak and Schmittgen, 2001). All the primers used for qRT-PCR are listed in Table 3.

2.3.5 Transformation methods

2.3.5.1 Preparation of competent *Escherichia coli* cells (Inoue *et al.*, 1990)

E. coli strain DH5α was inoculated in 50 ml SOB (0.5% yeast extract, 2% tryptone, 2.5 mM KCl, 10 mM NaCl, 10 mM $MgCl_2$, 10 mM $MgSO_4$) and incubated overnight at 37 °C as pre-culture. Next day the $O.D_{600}$ of 250 ml SOB was adjusted to 0.1 with the corresponding pre-culture and incubated at 37 °C until the $O.D_{600}$ reached 0.6. Following, the cells were incubated on ice for 10 min and centrifuged at 3000 rpm and 4 °C for 10 min. The pellet was resuspended in 80 ml ice-cold TB buffer (10 mM HEPES, 15 mM $CaCl_2$, 55 mM $MnCl_2$, pH 6.7) followed by 10 min incubation on ice and centrifugation. The cells were resuspended in 20 ml ice-cold TB buffer and gently mixed with 1.4 ml DMSO.

After 10 min incubation on ice, the cells were dispensed in 400 ml aliquots, frozen in liquid N_2 and stored at -80 °C.

2.3.5.2 Transformation of *Escherichia coli* (Inoue *et al*., 1990)

For transformation, the competent cells were thawed on ice and 5-10 µl DNA was mixed with 100 µl of the cells followed by 30 min incubation on ice, 1 min heat shock at 42 °C and 5 min incubation on ice again. Afterwards, 400 ml SOC (SOB with 20 mM glucose) was added and incubated for 15 min at 37 °C. The samples were spread on LB-plates containing 100 mg/l ampicillin and incubated overnight at 37 °C.

2.3.5.3 Transformation of *Saccharomyces cerevisiae* (Ito *et al*., 1983)

For transformation, the *S. cerevisiae* strains were cultivated in 10 ml YEPD medium overnight at 30 °C. On the following morning, 1 ml of the overnight culture was inoculated in 10 ml YEPD medium as main culture and incubated for 5 hr at 30 °C. Afterwards, the cells were centrifuged at 2000 rpm and 4 °C for 3 min and washed in 10 ml LiOAC/TE buffer (100 mM LiOAC, 10 mM Tris and 1 mM Na_2-EDTA). The centrifugation and washing steps were repeated twice. Afterwards, the cells were resuspended in 400 µl LiOAC/TE buffer. 200 µl of the cell suspension was mixed with 20 µl carrier DNA (single stranded salmon sperm DNA), 500 ng plasmid and 800 µl 50% PEG 4000 (polyethylene glycol). Afterwards, the cells were incubated for 30 min incubation at 30 °C and subjected to heat shock for 25 min at 42 °C. Then cells were centrifuged at 13000 rpm for 1 min, resuspended in 1 ml YEPD medium and incubated for 30 min at 30 °C. Finally, the cells were centrifuged at 13000 rpm for 1 minute, the remaining cells were spread on corresponding selective SC medium plates and incubated for 2-3 days at 30 °C.

2.3.5.4 Transformation of *Aspergillus fumigatus* (Yelton *et al*., 1984)

A. fumigatus strains were inoculated in 200 ml MM medium and grown on the shaker (180 rpm) overnight at 37 °C. The mycelia were filtered with sterile miracloth (Merck KGaA, Darmstadt, D) and washed with citrate buffer (50 mM Na-Citrate, 150 mM KCl, 580 mM NaCl, pH 5.5). Washed mycelia were incubated in 20 ml of vinoflow solution (600 mg Vinoflow and 300 mg Lysozyme in 20 ml citrate buffer) with slow shaking (60 rpm) for 1-2 hr at 30 °C. Afterwards, the protoplasts were harvested by filtering the solution through miracloth into 50 ml falcon, the total volume was brought to 50 ml with ice-cold STC1700 buffer (10 mM Tris pH 5.5, 35 mM NaCl, 50 mM $CaCl_2$, 1.2 M Sorbitol) and kept on ice for 5-10 min. After the centrifugation for 12 min at 4 °C, 2500 rpm, the protoplasts were resuspended in 50 ml ice-cold STC1700 buffer and centrifuged for 12 min at 4 °C, 2500 rpm. Subsequently, the supernatant were discarded and the protoplasts were resuspended in the leftover. By transferring the protoplasts to 15 ml tube, mixed with 3 μg linear DNA and incubated on ice for 25 min. PEG 4000 solution (10 mM Tris pH 7.5, 50 mM $CaCl_2$, 60% PEG 4000) was added to the samples in 250 μl, 250 μl, 850 μl by steps. The samples were mixed gently between step. Afterwards, the samples were incubated above ice for 20 min, the total volume was brought to 15 ml with ice-cold STC1700 buffer and centrifuged at 4 °C, 2500 rpm for 15 min. Finally, the samples were resuspended in leftover, added to two 5 ml top agar (LM medium supplemented with 1.2 M sorbitol and 0.7 % agar) and plated on selective protoplast plates (MM medium with the addition 0.6 M KCl and 2 % agar). The plates were incubated for 3-4 days at 37 °C.

2.3.6 Southern analysis

2.3.6.1 Probe preparation

To prepare the probe for Southern hybridization, the CPD-Star Kit (GE Healthcare Europe GmbH, Freiburg, D) was used according to user's manual.

100 ng DNA fragment and 1 µl 1:1000 diluted 1 kb DNA marker (Fermentas GmbH/Thermo Fisher Scientific GmbH, St. Leon-Rot/Schwerte, D) were mixed in a total volume of 10 µl. The DNA mixture was boiled at 95 °C for 5 min and transferred to ice immediately. Afterwards, the sample was mixed with 10 µl reaction buffer, 1 µl labeling reagent and 10 µl cross linker working solution (1:5 dilution) following by incubation for 30 min at 37 °C. The probe could be used immediately or stored at -20 °C.

2.3.6.2 Southern blot hybridization (Southern, 1975)

20 µg genomic DNA was digested overnight by a specific restriction enzyme. Afterwards, samples were loaded on an agarose gel and DNA was separated by electrophoresis. The gel was washed in 0.25 M HCl for 10 min, denaturing buffer (0.5 M NaOH, 1.5 M NaCl) for 25 min and neutralization buffer (0.5 M Tris pH 7.2, 1.5 M NaCl) on the shaker for 30 min, respectively. Afterwards, DNA was transferred to a Amersham Hybond-N nylon membrane (GE Healthcare Europe GmbH, Freiburg, D) by dry-blotting for 2 hr. Subsequently, the membrane was washed in 2X SSC (15 mM NaCl, 30 mM Na_3-Citrate, pH 7) twice and dried for 7 min at 70 °C. DNA was cross linked to a membrane by UV light exposure (λ= 254 nm) for 3 min on each side and the membrane was incubated in 15 ml pre-warmed hybridization solution for 1 hr at 55 °C. The DNA probe was added into hybridization buffer and incubated overnight at 55 °C. On the following day, the membrane was washed twice in 30 ml washing buffer I (1 mM $MgCl_2$, 3.5 mM SDS, 50 mM Na-Phosphate buffer, 150 mM NaCl, 2 M Urea, 0.2 % blocking reagents) for 10 min at 55 °C. Afterwards, the membrane was washed twice in 50 ml fresh washing buffer II (2 mM $MgCl_2$, 50 mM Tris Base, 100 mM NaCl, pH 10) for 10 min at RT. 0.5 ml detection reagent (CDP-Star, GE Healthcare Europe GmbH, Freiburg, D) was distributed on the membrane for 5 min and the membrane was incubated with Hyperfilm ECL (GE Healthcare Europe GmbH, Freiburg, D) for 30 min. The signal from the DNA probe was visualized on the film.

2.4 Protein methods

2.4.1 Protein isolation from *Saccharomyces cerevisiae*

To isolate proteins from yeast, strains were inoculated in SC-ura-leu medium and incubated overnight at 30 °C as pre-culture. On the following morning, 2 ml of pre-culture was added to new SC-ura-leu-met medium as main-culture and incubated for 6 hrat 30 °C. Afterwards, the cells were incubated on ice for 10 min and centrifuged for 5 min at 2000 rpm. The pellets were resuspended in 2 ml breaking buffer (1 mM Dithiothreitol (DTT), 100 mM Tris (pH8), 20 % glycerol) and centrifuged for 2 min at 2000 rpm. The supernatant was discarded, the cells were resuspended in 250 µl breaking buffer and transferred to a 1.5 ml tube. Glass beads were added into the tube and the sample was vortexed for 5 min at 4 °C. Then, 350 µl breaking buffer was added into the samples and the tubes were vortexed for shortly and centrifuged for 15 min at 13000 rpm. The supernatant was transferred to new 1.5 ml tube. Protein concentrations were measured at $O.D_{595}$ by Bradford assay (Bradford, 1976) using the Roti-Quant assay solution (Carl Roth GmbH and Co.KG, Karlsruhe, D).

2.4.2 β-galactosidase assay

The assays were performed as previously described (Braus *et al.*, 2003). Briefly, 200 µl Z-buffer (1 mM $MgSO_4$, 10 mM KCl, 40 mM NaH_2PO_4, 60 mM Na_2HPO_4) was added into a 96-well plate and 10 µl protein extract was mixed with Z-buffer and incubated for 5 min at 30 °C. Afterwards, 40 µl *ortho*-nitrophenyl-β-galactoside (oNPG) solution (10 mg/ml) was mixed with the samples at 30 °C. Then 100 µl Na_2CO_3 was added into the samples to stop β-galactosidase reaction. When the color of solution starts to turn yellow and the time was recorded. The samples were measured at 420 nm.

The activity of β-galactosidase was determined by the formula belowed (Rose and Botstein, 1983).

$$\text{Activity}\left(\frac{\text{nmole}}{\text{min}\cdot\text{mg}}\right) = \frac{\text{O.D}_{420}\times 0.35\ \text{nmole}}{0.0045\times\text{Protein concentration}\left(\frac{\text{mg}}{\text{ml}}\right)\times 0.01\ \text{ml}\ \times\text{Time (min)}}$$

2.4.3 Protein isolation from *Aspergillus fumigatus*

The *A. fumigatus* strains were grown for 24 h in 200 ml MM. The mycelium was harvested with miracloth filters and ground in liquid nitrogen. Protein crude extracts were obtained by mixing ground mycelium with B* buffer (300 mM NaCl, 100 mM Tris pH 7.0, 10 % glycerol, 2 mM EDTA, 0.02 % NP40, 2 mM DTT, 1 mM PMSF, 2 protease inhibitor pills/100 ml (Complete, EDTA-free, Roche Diagnostics GmbH, Mannheim, D). 400 µl B* buffer was added to 1 ml mycelium power and was vortexed for 4 min at 4 °C. Afterwards, the samples were centrifugated for 30 min at 13000 rpm, 4 °C. The supernatant was transferred to a new tube. The protein concentration was measured with the Nanodrop ND-1000 (Peqlab Biotechnologie GmbH, Erlangen, D).

2.4.4 GFP-Trap purification

The crude protein extracts of *A. fumigatus* strains were performed as previously described (Chapter 2.4.3). Crude extracts were mixed with 15 µl GFP-Trap beads (Chromo Tek GmbH, Planegg-Martinsried, D), which has been washed with B* buffer, and incubated for 2 h on a rotating machine at 4 °C. After the incubation, the beads were washed twice with 1.5 ml and 1 ml of B* buffer. After the centrifugation for 1 min at 4500 rpm at 4 °C, the supernatant was removed. The beads were resuspended with 40 µl 6 X loading dye (250 mM Tris

pH 6.8, 15 % β-mercaptoethanol, 30 % glycerol, 7 % SDS, 0.3 % bromophenol blue) and boiled for 6-8 min at 95 °C to separate the proteins from the beads.

2.4.5 Trypsin in-gel digestion

Trypsin digestion was performed as previously described (Shevchenko *et al.*, 1996; von Zeska Kress *et al.*, 2012). Briefly, the GFP-trapped samples were applied to a 12% SDS-PAGE and ran until the bands moved 1 cm into the separating gel. The samples were cut out from the gel and incubated with acetonitrile for 10 min at RT. Further, acetonitrile solution was removed and the gel pieces were dried in the SpeedVac Concentrator (Thermo Scientific GmbH, Dreieich, D). 150 µl of 10 mM DTT in 100 mM NH_4HCO_3 was added to the samples and incubated for 1 h at 56 °C. The DTT solution was removed and 150 µl of 55 mM iodoacetamide in 100 mM NH_4HCO_3 was added. The samples were incubated in the dark for 45 min at RT. Afterwards, the iodoacetamide solution was removed, the samples were washed with 150 µl 100 mM NH_4HCO_3 for 10 min and with 150 µl acetonitrile for 10 min. This washing step was repeated once and the samples were dried again at 60 °C. The samples were incubated with trypsin digestion buffer (1:20 sequencing grade trypsin (V5111, Promega GmbH, Mannheim, D) on ice for 45 min. Excessive buffer was removed and the gel pieces were incubated with 60 µl 25 mM NH_4HCO_3 overnight at 37 °C. The next day, the gel pieces were centrifuged for 1 min at 13000 rpm and the supernatant was collected. The gel pieces were incubated with 60 µl 20 mM NH_4HCO_3 for 10 min and the supernatant was also collected. Three elution steps were done. The gel pieces were incubated with 60 µl 50 % acetonitrile/5 % formic acid for 20 min, centrifugated for 1 min at 13000 rpm and the supernatant was collected. All collected supernatant were dried in the SpeedVac (Therrmo Fisher Scientific GmbH, Dreieich, D). The protein pellet was resolved in 20 µl 95 % H_2O/5 % acetonitrile/0.1 % formic acid and applied to LC/MS analysis. Particles and

precipitates were removed with Ultrafree-MC HV centrifugal filter units (Merck KGaA, Darmstadt, D) by centrifugation for 2 min at 10000 rpm.

2.4.6 Protein identification by mass spectrometry

Mass spectrometry analysis was performed as previously described (von Zeska Kress *et al.*, 2012; Harting *et al.*, 2013). Briefly, peptides in sample solution were trapped and washed with 0.05 % trifluoroacetic acid on an *Acclaim® PepMap 100* column (75 μm x 2 cm, C18, 3 μm, 100 Å, P/N164535, Thermo Scientific GmbH, Dreieich, D) at a flow rate of 4 μl/min for 12 min. Peptide separation was performed on an *Acclaim® PepMap RSLC* column (75 μm x 15 cm, C18, 3 μm, 100 Å, P/N164534, Thermo Scientific GmbH, Dreieich, D) running a gradient from 96 % solvent A (0.1 % formic acid) to 4 % solvent B (acetonitrile, 0.1 % formic acid) and to 50 % solvent B within 25 min at flow rate of 250 nl/min (solvents and chemicals: Fisher Chemicals).

Peptides eluting from the chromatographic column were on-line ionized by nano-electrospray using the Nanospray Flex Ion Source (Thermo Scientific GmbH, Dreieich, D) and transferred into the mass spectrometer. Full scans within the mass range of m/z 300-1850 were recorded by the Orbitrap-FT analyzer at a resolution of 60.000 at m/z 400. Peptides were fragmented by collision-induced decay in the LTQ Velos Pro linear ion trap. LC/MS method programming and data acquisition was performed with the softward *Xcalibur* 2.2 (Thermo Scientific GmbH, Dreieich, D).

Orbitrap raw files were analyzed with the Proteome Discoverer 1.4 software (Thermo Scientific, San Jose, Ca, USA) using the Mascot and Sequest search engines against the *A. fumigatus* protein database with the following criteria: peptide mass tolerance 10 ppm; MS/MS ion mass tolerance 0.8 Da, and up to two missed cleavages allowed. Methionine oxidation was considered as variable modification and carbamidomethylation was considered as fixed modification. For protein identification at least two different high ranking peptides

were required after validation against a decoy database had a false discovery rate of 0.01 or less. High peptide confidence and a minimum of two peptides per protein were used as result filters.

2.5 Phenotype characterization of *Saccharomyces cerevisiae*

2.5.1 Flocculation assay

The flocculation assay was performed as previously described (Kobayashi *et al.*, 1996). The strains carrying *AfusomA*, *ScFLO8* or empty vector were incubated in SC selective medium for one day at 30 °C on a rotating platform. Afterwards, the cell pellets were disrupted by adding 1 ml Na_2-EDTA (pH 8). The value of flocculation was determined by F = 1-B/A, where A is OD_{600} in solution without 0.1 % $CaCl_2$ and B is OD_{600} in the presence of 0.1 % $CaCl_2$.

2.5.2 Adhesion assay

To test whether the strains expressing *AfusomA* or *ScFLO8* can invade into agar surface, an adhesion assay was performed. The corresponding strains were streaked out on selective medium plates and incubated for 3 days at 30 °C. Afterwards, the plates were photographed (pre-washed) and washed gently in water until the negative control was washed away. Then, the plates (washed) were photographed again.

2.5.3 Pseudohyphal growth

To test the ability of diploid yeast strain to perform filamentous growth, a pseudohyphal growth test was performed. The corresponding strains carrying *ScFLO8* or *AfusomA* were streaked out on synthetic low ammonium dextrose (SLAD) plates which contain 0.15 % yeast nitrogen base without amino acid and

$(NH_4)_2SO_4$, 50 μM $(NH_4)_2SO_4$, 2 % glucose and 3% agar for 6 days at 30 °C. Afterwards, the plates were photographed.

2.5.4 Spotting assay

To test whether the expression of *AfusomA* and *ScFLO8* results in reduced growth, the spotting assay was performed. Strains were spotted in 10-times serial dilution either on SC-Ura or on SC-Ura-Met plates and incubated for 3 days at 30 °C. Then, the plates were photographed.

2.6 Phenotype characterization of *Aspergillus fumigatus*

2.6.1 Growth test

Harvested conidia were diluted in NaCl-Tween solution (0.9 % NaCl, 0.02 % Tween 20) to reach 10^5 spores/ml using a Thoma counting chamber (Paul Marienfeld GmbH and Co. KG, Lauda-Königshofen, D). 5 μl of this spore solution, which contained 500 spores, was spotted on MM plates and incubated for 5 days at 37 °C. The colony diameter was measured. For adhesin deletion strains, growth test was also performed on MM with 1.2 M sorbitol and MM with 0.6 M KCl. For two conditional expression *AfusomA* mutants and *Xyl-AfusomA* strain was spotted on MM supplemented with 1 % xylose, while *Tet-AfusomA* mutant was performed on MM with 5 mg/l doxycycline.

2.6.2 Adhesion assay

To test whether AfuSomA or putative adhesins are responsible for adherence to plastic, the adhesion assay was performed. The harvested conidia of the corresponding strains were diluted in MM medium to reach 10^5 spores/ml. 1 ml of this spores solution was added to 12-well culture plate (Greiner Bio-One GmbH, Frickenhausen, D) and incubated for 24 h at 37 °C. Afterwards, the

medium was discarded by pipetting and the wells were washed with phosphate buffer saline (PBS) three times. The samples were dyed with 0.01% crystal violet and photographed. Addition of 5 mg/l doxycycline was applied to activate the expression of the *Tet-AfusomA* mutant.

2.7 Microscopy

To analyze *A. fumigatus* asexual development, strains were grown on an agar-coated slide with a thin layer of MM agar or MM agar with 5 mg/l doxycycline for 28 h and then observed with the Axiolab microscope (Carl Zeiss GmbH, Jena, D) at 400-fold magnification. For aerial hyphae visualization, the corresponding strains were grown on agar slide with either MM agar or MM agar containing 5 mg/l doxycycline for 28 h, and observed with SZX-ILLB2-200 binocular microscope (Olympus GmbH, Hamburg, D). Picture were obtained with a CS30 digital camera (Olympus GmbH, Hamburg, D) and with the cellSens software (Olympus GmbH, Hamburg, D).

2.8 Egg infection model

2.8.1 Conidia preparation

The strains were grown on malt extract agar for 7 days at 37 °C. On the day of infection, the conidia were freshly harvested in PBS with 0.1 % Tween 20 solution. In order to perform egg infection, the harvested conidia were first diluted to 10^5 spore/ml in PBS-0.1 % Tween 20 solution and then diluted to 10^4 spore/ml in PBS solution for infection. 100 µl of final spore solution was used to infect an egg. The wild type and *Tet-AfusomA* mutant were grown on malt extract agar with 5 mg/l doxycycline.

2.8.2 Infection

The egg infection model was performed as described (Jacobsen *et al.*, 2010; Jacobsen *et al.*, 2012). Fertilized eggs were obtained from a farm before incubation. Eggs were incubated for 10 days (day 0 to day 9) in an egg incubator TC 2010F (J.Hemel Brutgeräte GmbH and Co. KG, Verl, D) with 60 % humidity at 37 °C. From day 3 until the day for infection, the eggs were turned lengthways every 6 h. At day 9, embryonic development and survival of incubated eggs were checked. In viable eggs, the blood vessel pattern of the chorioallantoic membrane (CAM) and natural air space could be visualized by candling eggs in a darkened box. The non-viable eggs were discarded. Viable eggs were numbered and the air cell was marked with a pencil.

At day 10 (infection day), conidia of the corresponding strains were harvested and counted. 20 eggs were used to be inoculated with one strain. To inoculate the spore solution, two holes were made on the shell where one hole is at air cell and the other is on the lengthwise side (Figure 9). The air in the air cell was removed by rubber bulb and artificial air space was generated simultaneously. 1000 spore in 100 µl PBS solution was inoculated through the hole at the lengthways side. Afterwards, both holes of the egg were sealed by paraffin and eggs were moved back into the incubator. Survival of eggs was checked once to twice a day until day 17. At day 17, surviving eggs were killed by freezing.

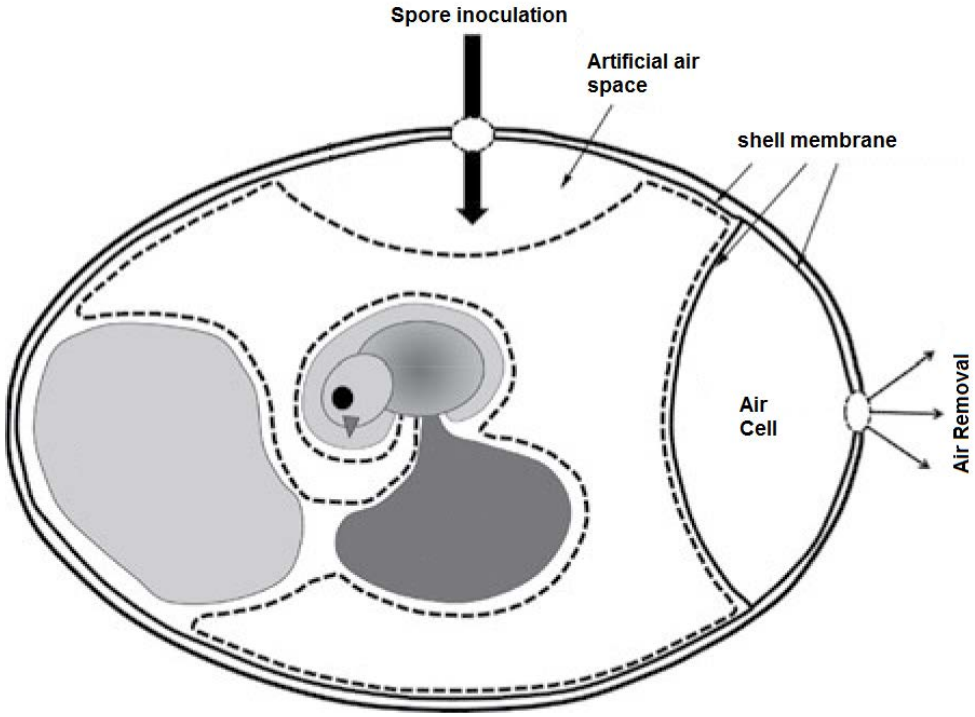


Figure 9. Scheme of egg infection.

In order to infect an egg, two holes were created. One is on the side of the air cell and the other is on the lengthwise side. When the air was removed from the air cell, the artificial air space was constructed. The spore solution of the corresponding strain was inoculated into the artificial air space (modified from (Jacobsen *et al.*, 2012)).

3 Results

3.1 The *FLO8* homolog of *Aspergillus fumigatus* complements morphological defects in Δ*flo8* yeast

3.1.1 Identification of *FLO8* homolog in *Aspergillus fumigatus*

Flo8 is a transcription factor for yeast dimorphism in *Saccharomyces cerevisiae* and *Candida albicans*. This morphological transition between yeast and hyphal forms is required for adhesion and virulence (Kobayashi *et al.*, 1996; Liu *et al.*, 1996; Cao *et al.*, 2006; Mayer *et al.*, 2013). Adherence to the host cells is an important step for pathogenesis, but the knowledge is limited in filamentous fungi. The *FLO8* homolog, *SOM1*, in the filamentous fungus *Magnaporthe oryzae* complements the adhesion in *S. cerevisiae* and is required for plant pathogenicity in rice (Yan *et al.*, 2011). This indicates that Flo8/Som1 protein may be responsible for adherence and virulence in filamentous fungi.

To identify the *FLO8/SOM1* homolog in *Aspergillus fumigatus*, the amino acid sequences of Flo8 of *S. cerevisiae* and *C. albicans* were used for blast research. The AfuSomA (7G02260) showed 15.7 % and 20.5 % identity to the Flo8 of *S. cerevisiae* and *C. albicans*, respectively. Comparing the homologous proteins in filamentous fungi, AfuSomA shared 39.1, 36.8, 75.6, and 41.7 % identity to *M. oryzae* Som1, *Neurospora crassa* Som1, *Aspergillus nidulans* Som1 and *Fusarium oxysporum* Som1. However, further analysis showed that AfuSomA is closer related to Flo8 in yeasts than to Som1 in filamentous fungi (Figure 10).

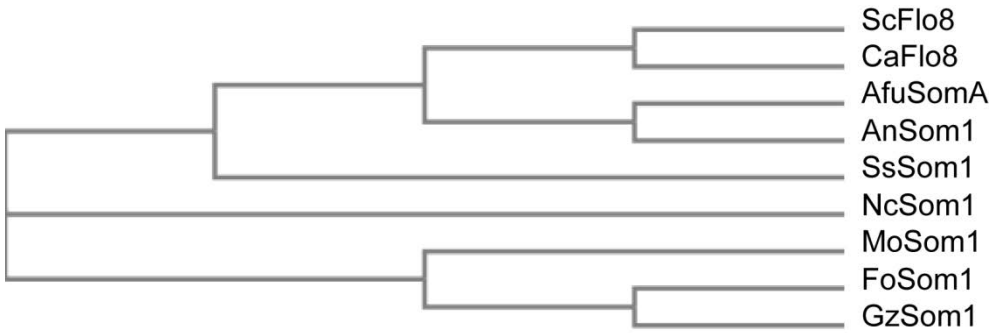


Figure 10. AfuSomA might share the same ancestor with Flo8.

Phylogenetic tree of Flo8/Som1 family members. The phylogenetic tree was predicted by ClustalW2 at European Molecular Biology Laboratory – European Bioinformatics Institute (www.ebi.ac.uk). Abbreviations and accession numbers are as follows: ScFlo8, *Saccharomyces cerevisiae* Flo8 (NP_011034); CaFlo8, *Candida albicans* Flo8 (XP_712106); AfuSomA, *Aspergillus fumigatus* SomA (XP_746706); AnSom1, *Aspergillus nidulans* Som1 (XP_682356); SsSom1, *Sclerotinia sclerotiorum* Som1 (XP_001598877); NcSom1, *Neurospora crassa* Som1 (AAF75278); MoSom1, *Magnaporthe oryzae* Som1 (ELQ39413); FoSom1, *Fusarium oxysporum* Som1 (EWZ81375); GzSom1, *Gibberella zeae* Som1 (XP_382826).

Using computational tools conserved functional domains were predicted (Figure 11). The Flo8/Som1 proteins share with AfuSomA (7g02260) the LUFS domain, which contains a LisH (Lis homology) motif for protein dimerization and tetramerization at the N-terminus. In addition to the LisH domain, there is a conserved nuclear localization signal (NLS) PSPSKRPRLE, which is shared by the representatives of filamentous fungi (Figure 11). Both the LisH domain and the NLS have been shown to be responsible for developmental regulation and virulence in *M. oryzae* (Yan *et al.*, 2011).

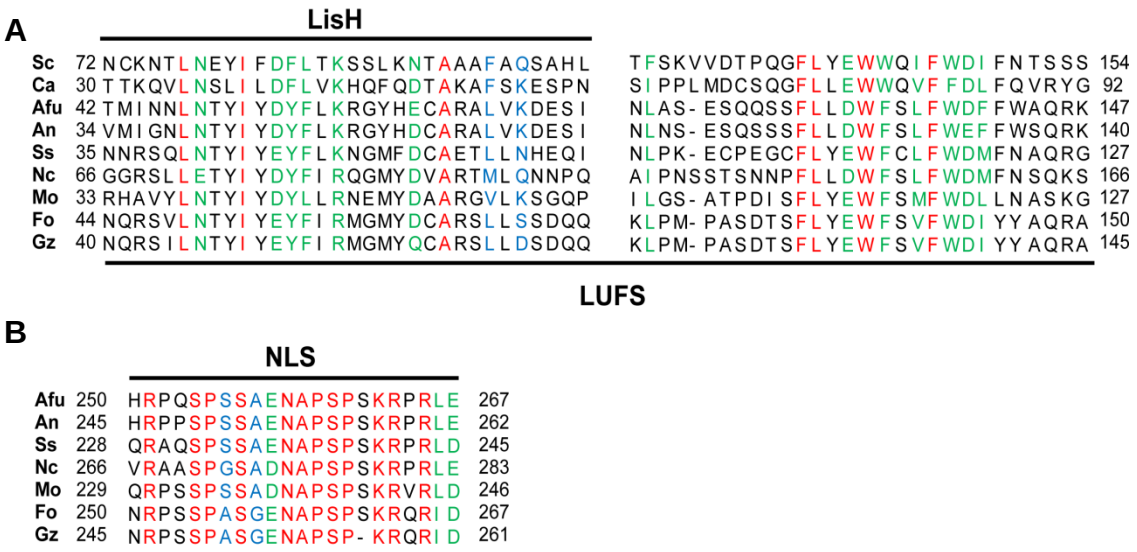


Figure 11. Partial sequence alignment of Flo8/Som1 proteins.

(A) Sequence alignment of the LUFS and LisH domains of Flo8/Som1 homologs. (B) Sequence alignment of predicted NLS of Som1 proteins in filamentous fungi. Multiple alignment was performed by Clustal Omega at European Molecular Biology Laboratory – European Bioinformatics Institute (www.ebi.ac.uk). NLS was predicted at cNLS mapper (http://nls-mapper.iab.keio.ac.jp/). Abbreviation are as follows: Sc, *Saccharomyces cerevisiae*; Ca, *Candida albicans*; Afu, *Aspergillus fumigatus*; An, *Aspergillus nidulans*; Ss, *Sclerotinia sclerotiorum*; Nc, *Neurospora crassa*; Mo, *Magnaporthe oryzae;* Fo, *Fusarium oxysporum*; Gz, *Gibberella zeae*. Identical residues are highlighted in red, highly consensus residues in green and modestly consensus residues in blue. Numbers indicate the amino acid position of the first and the last residue.

The *AfusomA* exons were identified by comparing the predicted mRNA sequence obtained from the *Aspergillus* Genome Database (AspGD http://www.aspergillusgenome.org) with cDNAs which were amplified from the total mRNA of wild type (AfS35) strain. The amplified *AfusomA* cDNAs were cloned into plasmid (pME2787) for sequencing. Sequencing of the resulting plasmid revealed that *AfusomA* carries five exons of a size of 486 bp, 152 bp, 1279 bp, 267 bp and 171 bp (pME4192) resulted in a deduced protein of 784 amino acids with a molecular weight of 84.59 kDa (Figure 12). An additional splice variant (pME4193) contained six exons with an additional small intron of 54 bp within exon 3. This resulted in a smaller protein of 766 amino acid. Both splice variants of *AfusomA* were identified in wild type strain grown for 20 h

(Figure 12). The multiple splice variants were also found in Som1 in *N. crassa* and *M. oryzae* (Yan *et al.*, 2011; Broad Institute).

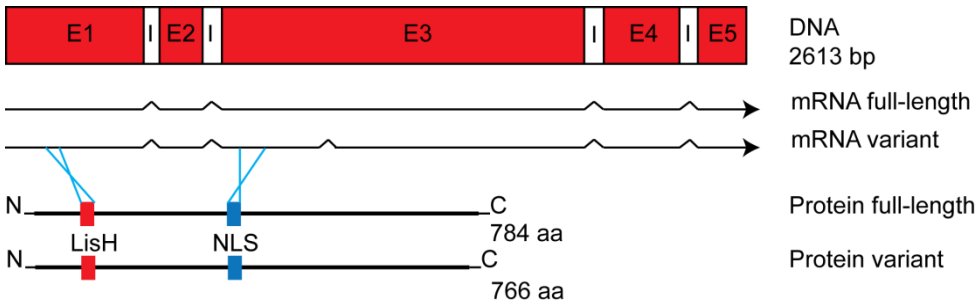


Figure 12. Structural organization of *AfusomA*.

Genomic structure of *AfusomA*, two mRNA splice variants and the deduced protein with LisH and predicted NLS motifs. The LisH (red) and NLS (blue) are located in exon 1 (E1) and exon 3 (E3), respectively. Abbreviation are as follows: E, exon; I, intron.

3.1.2 High expression levels of *AfusomA* inhibit growth and could not complement the defects in adhesive growth in Δ*flo8* yeast

Adhesive and filamentous growth in *S. cerevisiae* can be activated by limitation of ammonium or fermentable sugars such as glucose in the medium (Gimeno *et al.*, 1992; Cullen and Sprague, 2000). In order to test whether AfuSomA could complement either adhesive or pseudohyphal growth in Δ*flo8* yeast mutant, both splice variants were cloned into the plasmid (pME2787) carrying the *MET25* promoter. The normally high expression levels from this plasmid are repressed when methionine is present in the medium (Johnston and Davis, 1984; Sangsoda *et al.*, 1985). The plasmids carrying the two spliced variants were transformed into in yeast Δ*flo8* haploid strain BY4742. Then heterogeneous expression of *AfusomA* gene was performed in medium without methionine to activate its expression. AfuSomA was examined whether it can complement the defect in adhesive growth. In medium lacking methionine, neither *AfusomA* nor its splice variant (pME4194 and pME4195) could complement the invasive growth (cell-surface adhesion) on solid agar or

flocculation (cell-cell adhesion) in liquid medium in the Δ*flo8* haploid mutant strain (BY4742). The plasmids carrying *ScFLO8* (pME4197), which is also regulated by *MET25* promoter, was used as positive control (Figure 13). The empty vector pME2787 was used as negative control.

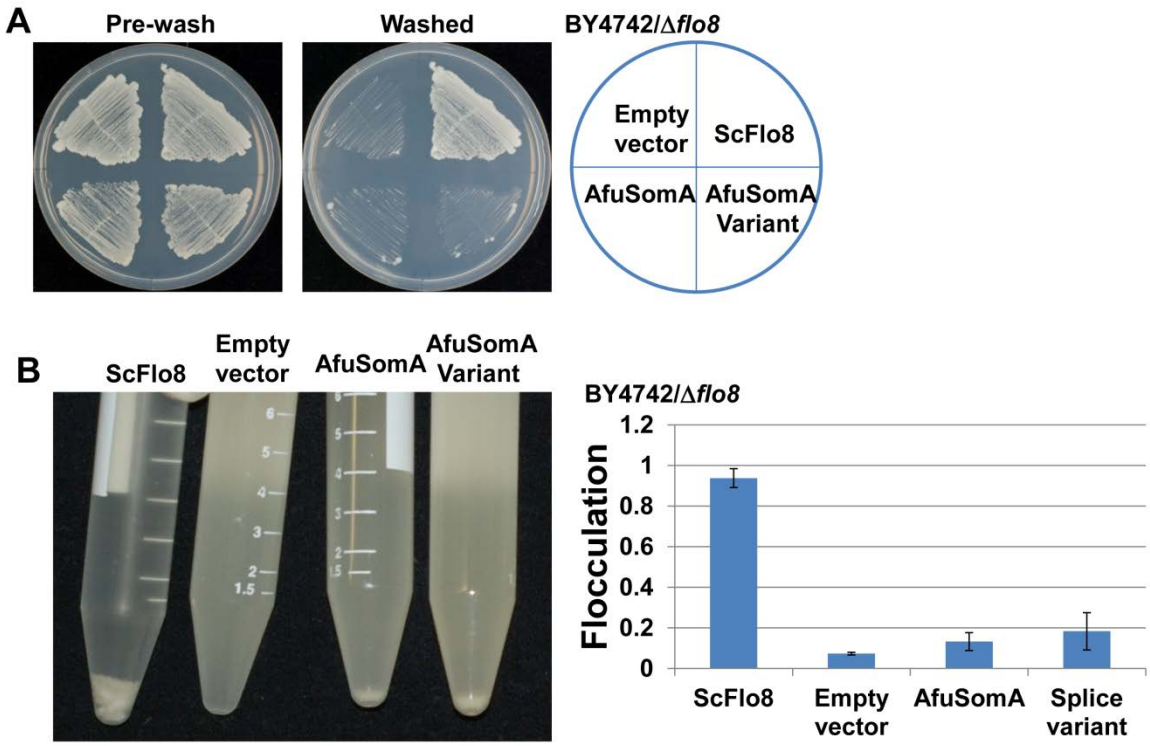


Figure 13. High expression levels of *AfusomA* can not complement the defect in adhesive growth in Δ*flo8* yeast.

(A) Invasive growth defects of Δ*flo8* haploid mutant (BY4742) were not rescued by expressing either AfuSomA (pME4194) or its splice variant (pME4195). Representative pictures show that strains expressing both *AfusomA* variants are washed away on Sc-Ura-Met plates. The experiments were carried out in triplicate. Strains, expressing ScFlo8 (pME4197) and empty vector (pME2787) were used as positive and negative controls. Strains were grown on SC-Ura-Met plates for 3 days at 30 °C, and the plates were photographed before and after washing under water tape. (B) AfuSomA and its splice variant revealed little flocculation at the bottom of the tubes compared to the positive control ScFlo8. The empty vector was used as a negative control. Flocculation quantification indicated that AfuSomA and splice variant showed similar flocculation rate with the empty vector. Strains were grown in 10 ml Sc-Ura-Met medium for one day. Graph indicates mean ± standard error and the experiments were performed in triplicates.

High expression levels of heterogeneous protein might be toxic to *S. cerevisiae* (Mumberg *et al.*, 1994). It was observed that both strains containing AfuSomA were growing less efficient. Therefore, the growth test was performed to determine the toxicity of both AfuSomA and its spliced variant in yeast. Strains carrying AfuSomA, AfuSomA splice variant, ScFlo8 and empty vector were grown on plates either with or without methionine. As shown in Figure 14, there is no significant difference between strains carrying AfuSomA, AfuSomA splice variant and ScFlo8 on plates with methionine (low expression levels). In contrast, growth inhibition was observed in strain carrying either AfuSomA or the splice variant on the plates without methionine (high expression levels). This result indicates that loss of adhesion or flocculation might be due to slow growth rate by high expression levels of AfuSomA in yeast.

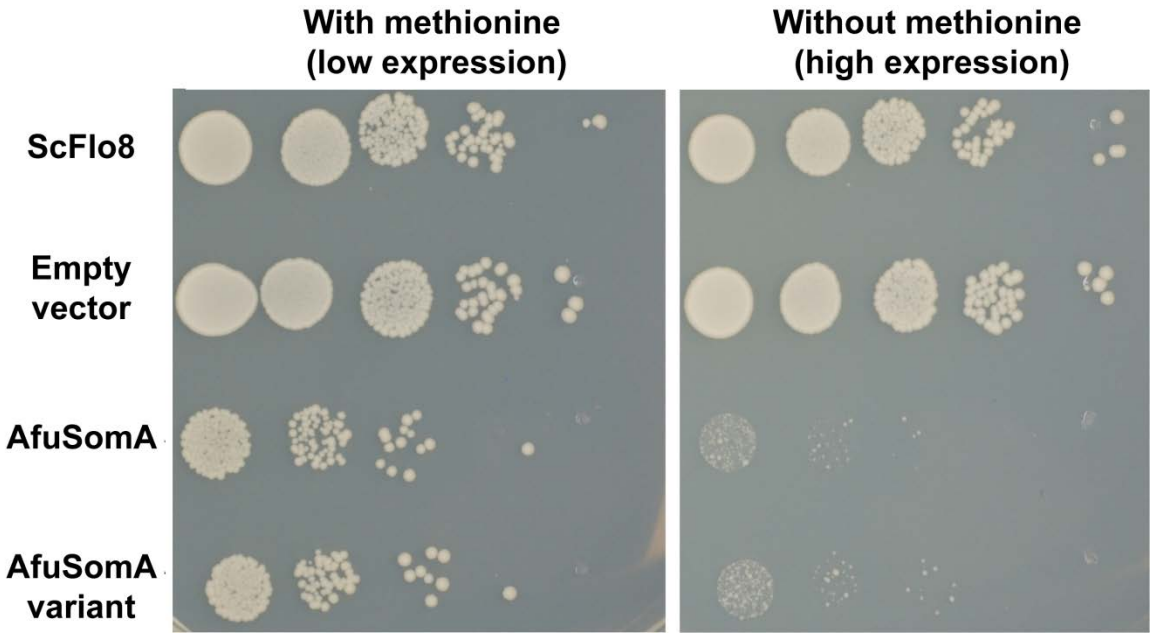


Figure 14. High expression levels of *AfusomA* results in growth inhibition in Δ*flo8* yeast.

ScFlo8, AfuSomA or AfuSomA splice variant were expressed under *MET25* promoter in the haploid Δ*flo8* strain BY4742. Empty vector (pME2787) was used as negative control. Strains were spotted in 10-fold dilutions either on Sc-Ura (with methionine) at low expression level or on Sc-Ura-Met (without methionine) at high expression level. Plates were incubated for 3 days at 30 °C and photographed.

3.1.3 Low expression levels of *AfusomA* complements the morphological defects in Δ*flo8* yeast

Since *MET25* promoter has low expression levels of the downstream gene in the presence of methionine (Mumberg *et al.*, 1994). Heterogeneous expression of *AfusomA* gene was performed on plates or in liquid medium with methionine to allow the investigation of invasive growth or flocculation in haploid Δ*flo8* mutant. Low expression levels of both *AfusomA* and its splice variant (pME4194 and pME4195) could rescue invasive growth in the Δ*flo8* haploid mutant on solid agar as well as flocculation in liquid medium similar to the ScFlo8 (pME4197) (Figure 15).

Apart from adhesive growth in haploid yeast, AfuSomA was examined whether it can complement pseudohyphal growth in diploid Δ*flo8* yeast. Plasmids carrying *AfusomA* (pME9194), *ScFLO8* (pME9196) and the empty vector (pME2787) were transformed into the Δ*flo8* diploid strain (RH2660). The wild type (RH2656) was transformed with the empty vector as positive control. Expression of *AfusomA* and *ScFLO8* in Δ*flo8* diploid strain restored pseudohyphal growth (Figure 16). The data from haploid and diploid Δ*flo8* mutants support that the AfuSomA and ScFlo8 can fulfill similar cellular functions in yeast and might share a common ancestor gene with *M. oryzae* Som1 protein which was also shown to complement pseudoyphal growth in diploid yeast (Yan *et al.*, 2011).

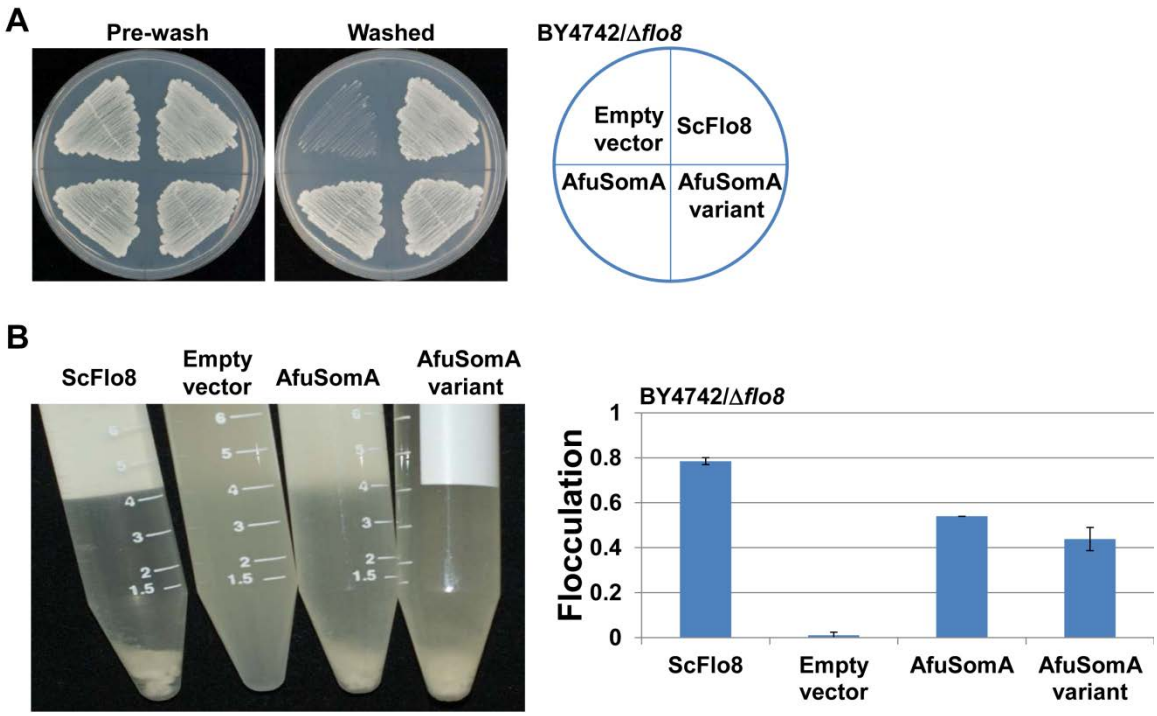


Figure 15. Low expression levels of *AfusomA* complements the defects in adhesive phenotypes in haploid Δ*flo8* yeast.

(A) Invasive growth defects of Δ*flo8* haploid mutant (BY4742) were rescued by expressing either AfuSomA (pME4194) or its splice variant (pME4195). Representative pictures show that both AfuSomA variants expressing strains are not washed away on Sc-Ura plates. The experiments were carried out in triplicate. Expression ScFlo8 (pME4197) and empty vector (pME2787) were used as positive and negative controls. Strains were grown for 3 days at 30 °C, and the plates were photographed before and after washing under water tape. (B) AfuSomA and its splice variant revealed similar levels of flocculation at the bottom of the tubes in comparison to strains carrying ScFlo8 as positive control. Empty vector (pME2787) was used as negative control. Flocculation quantification indicated that AfuSomA and the splice variant showed similar flocculation with ScFlo8. Strains were grown in 10 ml Sc-Ura medium for one day. Graph indicates mean ± standard error and the experiments were performed in triplicate.

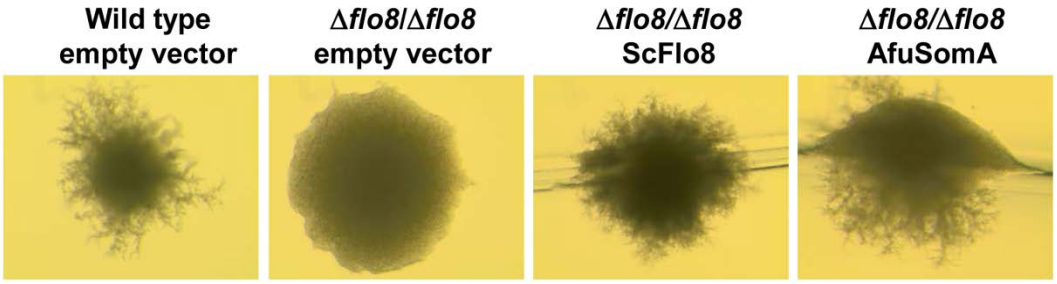


Figure 16. AfuSomA restores pseudohyphal growth in Δ*flo8* diploid yeast mutant.

Pseudohyphal growth of diploid Δ*flo8* mutant (RH2660) was rescued by expressing *AfusomA* (pME4194) and *ScFLO8* (pME4197). Empty vector (pME2787) was used as negative control. Strains were grown on SLAD for 6 days at 30 °C and photographed. Wild type (RH2656) transformed with empty vector (pME2787) was used as positive control. Experiments were carried out in triplicates.

3.1.4 AfuSomA and ScFlo8 recognize similar promoter sites for *ScFLO11* expression

ScFlo8 is a transcription factor, which binds and starts transcription at the *ScFLO11* promoter expressing the flocculin Flo11 (Bester *et al.*, 2006; Fichtner *et al.*, 2007). Flo11 mediates adhesion and represents the key determinant for haploid invasive growth or diploid pseudohyphal development in laboratory yeast strains where other *FLO* genes are silenced (Brückner and Mösch, 2011). ScFLO11 comprises one of the largest yeast promoters where ScFlo8 represents only one out of numerous transcription factors repressing or activating transcription (Brückner and Mösch, 2011). AfuSomA was examined whether it complements the adhesion in Δ*flo8* yeast mutants by activating *ScFLO11* gene expression (Figure 15 and 15). We performed β-galactosidase assays with the 3 kb *ScFLO11* promoter fused to the bacterial *LacZ* reporter gene (Rupp *et al.*, 1999). The reporter plasmid (pME2167) was co-transformed with the *AfusomA* expressing plasmids (pME4192 or pME4193), which are regulated under *MET25* promoter, into Δ*flo8* yeast strain (Y16870). Plasmids carrying the *ScFLO8* (pME4196) or the empty vector (pME2786) were co-transformed with reporter plasmid into Δ*flo8* yeast strain as positive and negative controls. To determine

the activity of β-galactosidase, transformed strains were grown in Sc-Ura-Leu medium overnight as pre-culture. Next day, samples were inoculated into Sc-Ura-Leu-Met medium for 6 h to induce the expression of either *AfusomA* or *ScFLO8*. Both AfuSomA and its splice variant showed significantly increased *ScFLO11* promoter driven *LacZ* activity in comparison to the mutant strain transformed with the empty plasmid (pME2786) (Figure 17).

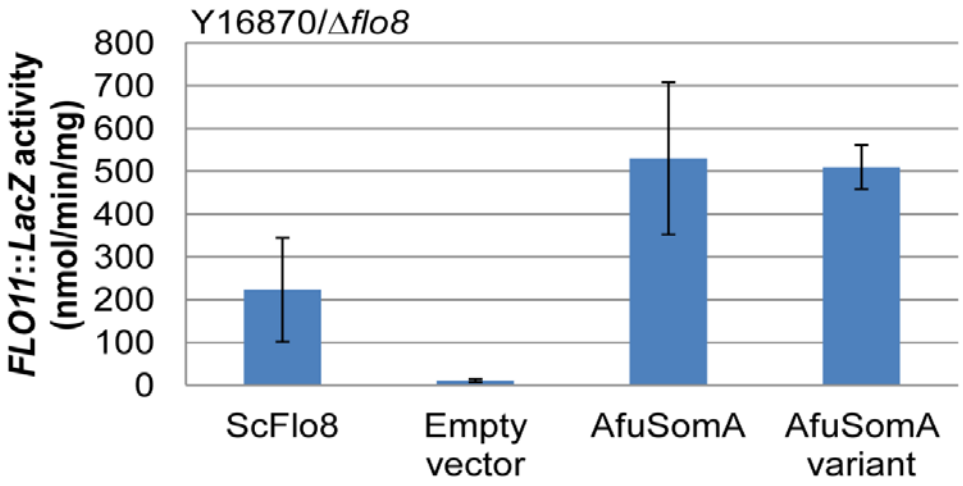


Figure 17. AfuSomA and ScFlo8 activate *ScFLO11* expression.

Expression of *ScFLO11::LacZ* was performed in haploid Y16870 strain (Δ*flo8*). Co-transformation of pME2167 carrying the *ScFLO11::LacZ* which encodes bacterial β-galactosidase, with plasmid harboring ScFlo8, AfuSomA, AfuSomA splice variant or the empty vector pME2786 was performed. Strains were grown in Sc-Ura-Leu medium as pre-culture, then the samples were inoculated into Sc-Ura-Leu-Met medium as main culture for 6 h before determination of the β-galactosidase activities were determined. Graph indicates mean ± standard errors and experiments were repeated in triplicates.

AfuSomA and its splice variant showed a difference in β-galactosidase activities compared to ScFlo8. Therefore, we took a more detailed look at the *ScFLO11* promoter to determine whether AfuSomA and ScFlo8 bind to similar regions of *ScFLO11* promoter. A set of 14 reporter constructs which contain 400 bp *ScFLO11* promoter fragments that overlap by 200 bp upstream of the *CYC1::lacZ* fused gene. This gene is comprised of *E. coli lacZ* gene and *S. cerevisiae* iso-1-cytochrome c (*CYC1*) gene (Rupp *et al.*, 1999). The *CYC1*

fragment contains TATA box for expression of *LacZ* in yeast (Guarente and Ptashne, 1981) (Figure 18A). All transformants were first grown in Sc-Ura-Leu and transferred to Sc-Ura-Leu-Met to activate the expression of *AfusomA* and *ScFLO8.* As shown in Figure 18B, two promoter regions were affected by both ScFlo8 and AfuSomA. Comparison of Figure 18A and 18B indicated that these two regions are located at 1.8 kb and 1.2 kb upstream of the start codon of *ScFLO11*. AfuSomA seems to recognize two additional regions located at 1.4 kb and 1 kb upstream of the *ScFLO11* open reading frame. These data corroborate that AfuSomA and ScFlo8 share molecular functions in recognizing and controlling similar regions of the *ScFLO11* promoter and hence complemented adhesion and filamentous growth in Δ*flo8* yeast strains.

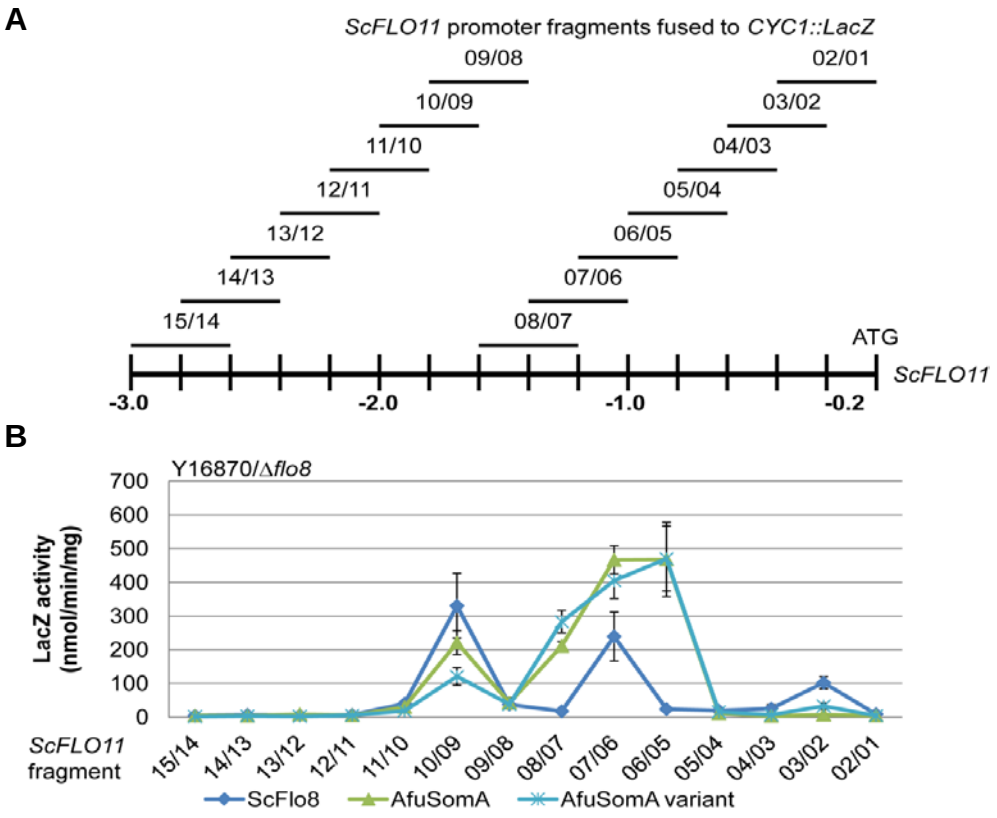


Figure 18. AfuSomA and ScFlo8 act through similar regions of *ScFLO11* promoter.

(A) Schematic overview of 14 different 400 bp constructs of the *ScFLO11* promoter region fused to *CYC1::LacZ* reporter which is a hybrid genes of *E. coli lacZ* gene and *S. cerevisiae* iso-1-cytochrome c (*CYC1*) gene (Guarente and Ptashne, 1981; Rupp *et al.*, 1999). (B) The expression of *LacZ* gene fused to different *ScFLO11* promoter fragments was performed in Y16870 strain (Δ*flo8*). Plasmids carrying ScFlo8, AfuSomA or AfuSomA splice variant, which were expressed under *MET25* promoter, were co-transformed with one of the 14 plasmids containing different 400 bp fragments of the *ScFLO11* promoter fused to the *CYC1::LacZ* reporter gene. *ScFLO11* promoter fragment dependent β-galactosidase activities of the strain harboring either ScFlo8 (diamonds), AfuSomA (triangles) or AfuSomA splice variant (asterisks) are indicated as means ± standard errors. Experiments were repeated for three times. Strains were grown in 10 ml Sc-Ura-Leu medium as pre-culture, then 1 ml of each sample was inoculated into Sc-Ura-Leu-Met medium as main culture for 6 h before the β-galactosidase activities were determined.

3.2 AfuSomA is required for asexual development in *Aspergillus fumigatus*

3.2.1 Deletion of the *AfusomA* gene blocks asexual development at aerial hyphae

The Som1 proteins have been shown to be required for asexual/sexual development in *M. oryzae* and *A. nidulans* (Lee *et al.*, 2005; Yan *et al.*, 2011). To verify the function of AfuSomA in cellular development, the Δ*AfusomA* mutant strain was designed using the six recyclable marker system (Hartmann *et al.*, 2010) (Figure 19A). The deletion fragment was constructed in plasmid pME4188 and transformed into the *A. fumigatus* strain AfS35. In this strain the Ku70 component of nonhomologous end-joining machinery was deleted to facilitate the homologous recombination in *A. fumigatus* (Krappmann *et al*., 2006). Homologous integration was verified by Southern hybridization with a probe detecting the 5' flanking region of *AfusomA.* Genomic DNA of wild type and *AfusomA* deletion strains were digested with *Xba*I restriction enzyme and showed a signal at about 4.5 kb for the wild type strain and 5.1 kb for the Δ*AfusomA::ptrA* mutant (AfGB72) (Figure 19B). The deletion of *AfusomA* resulted in a distinct defect in asexual spore formation. The mutant could not be propagated by inoculating asexual spores but through small agar pieces, which had to be cut from previous plates for propagation. Cultivation on additional minimal medium (MM) plates revealed slow growth (2.7 mm colony radius/day) of the Δ*AfusomA* strain (AfGB72) in comparison to wild type (6.7 mm/day) combined with impaired asexual sporulation (Figure 20A). The defect in asexual spore formation of the Δ*AfusomA* mutant was also observed in a Δ*brlA* strain except of the growth retardation (Twumasi-Boateng *et al.*, 2009) (Figure 20A). The observed Δ*AfusomA* phenotypes were verified by complementation with the respective wild type gene (Figure 19A). To perform the complementation, the Δ*AfusomA* mutant was grown on MM plate with 1% xylose to remove the resistance marker resulting in Δ*AfusomA::six* strain (AfGB77) (Figure 19A). Southern hybridization with a probe detecting the 5' flanking region of *AfusomA*

was used to examine the loss of resistance marker. Genomic DNA of wild type and Δ*AfusomA::six* strain were digested with *Xba*I restriction enzyme and showed a signal at about 4.5 kb for the wild type strain and 5.1 kb for the Δ*AfusomA::six* mutant (AfGB77) (Figure 19B). This strain was transformed with complementation fragment from pME4190 to yield the complemented strain. Southern hybridization with a probe detecting the 3' flanking region of *AfusomA* was performed to verify the complementation. Genomic DNA of the wild type and the complemented strains were digested with *Eco*RI restriction enzyme and showed a signal at about 3.8 kb for the wild type strain and 2.2 kb for the complemented mutant (AfGB73) (Figure 19C). The complemented strain (AfGB73) had improved growth rates (5.2 mm/day in comparison to 2.7 mm/day in the mutant) and normal conidiation, which is indistinguishable from the wild type (Figure 20A).

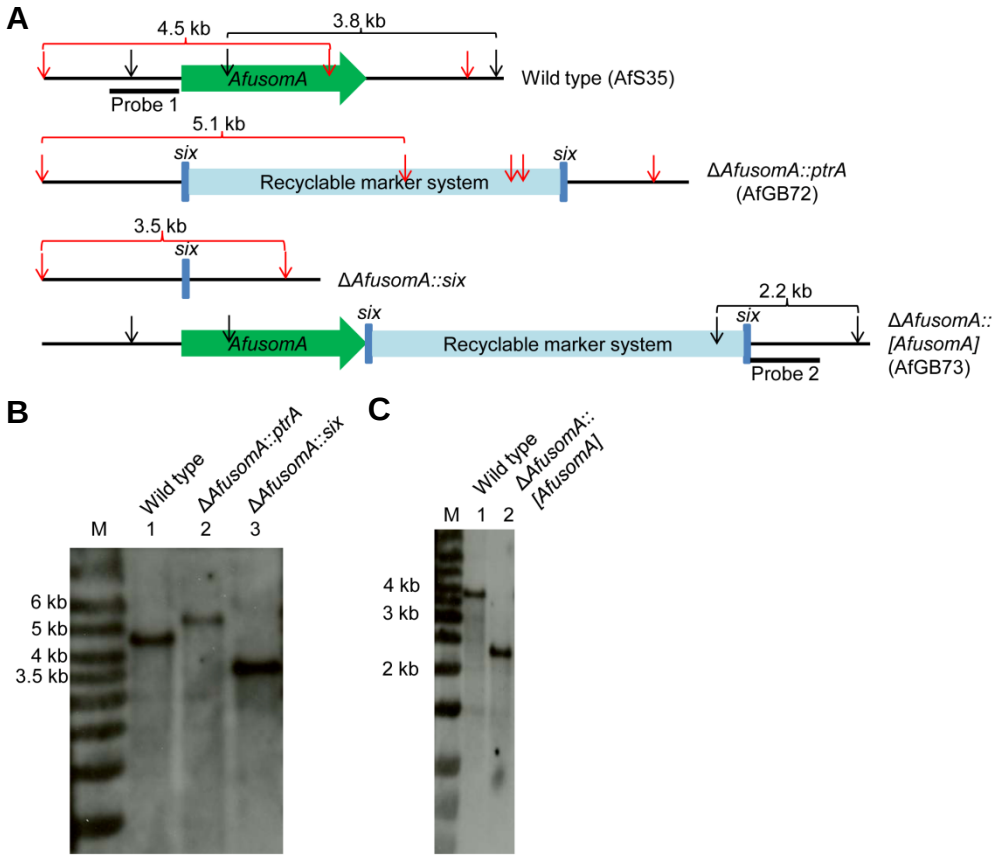


Figure 19. Scheme of the deletion and the complementation construct for *AfusomA* and Southern hybridization.

(A) Wild type (AfS35) represents the original locus of *AfusomA* gene in the chromosome. Following are the Δ*AfusomA::ptrA* (AfGB72) strain, Δ*AfusomA*::*six* (AfGB77) strain, whose resistance marker is removed, and the complemented strain Δ*AfusomA*:: *[AfusomA]* (AfGB73). Green arrow represents *AfusomA* gene and blue rectangle is the recyclable marker system containing pyrithiamine resistance (*prtA*) cassette. The blue vertical bars present *six* recombination sites for marker recycling. Probes used for southern hybridization are indicated as horizontal black bars. Red and black vertical arrows indicate *Xba*I and *Eco*RI restriction site for DNA digestion, respectively. (B) Southern hybridization of wild type (Lane 1), Δ*AfusomA*::*ptrA* (Lane 2) and Δ*AfusomA*::*six*, whose marker is removed (Lane 3). Genomic DNA was digested with *Xba*I restriction enzyme and 5' flanking region of *AfusomA* was used as probe 1 for hybridization. The expected size of wild type, Δ*AfusomA*::*ptrA* and Δ*AfusomA*::*six* is 4.5 kb, 5.1 kb and 3.5 kb, respectively. (C) Southern analysis of wild type (Lane 1) and the complemented strain (Lane 2). Restriction digestion was performed with *Eco*RI and signal detection was carried out with 3' flanking region (probe 2). The expected size of wild type and the complemented strain are 3.8 kb and 2.2 kb, respectively.

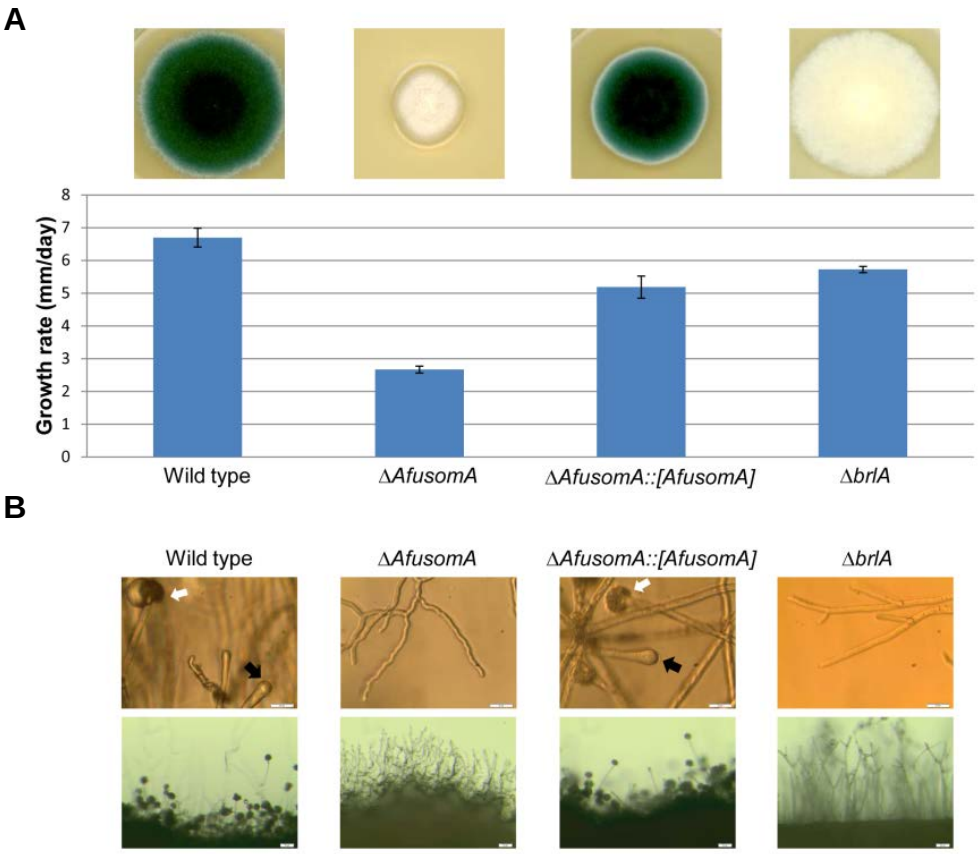


Figure 20. AfuSomA is required for growth and conidiation.

Colony morphology and growth rate of wild type (AfS35), the Δ*AfusomA* mutant (AfGB72), the complemented strain Δ*AfusomA::[AfusomA]* (AfGB73) and Δ*brlA* mutant (A1176). All strains were grown on MMs plate for 5 days at 37 °C. Values in the graph are indicated as means ± standard error. (B) Morphology of conidiation of wild type, the Δ*AfusomA* mutant, the complemented strain Δ*AfusomA::[AfusomA]* and Δ*brlA* mutant. Upper panel: Strains were grown on MM agar-coated slide for 28 h at 37 °C. In wild type and the complemented strains, white arrows indicates conidiophores and black arrows represent the vesicle for sporulation. Neither vesicles nor conidiophores were found in Δ*AfusomA* and Δ*brlA* mutant. Lower panel: Strains were grown on MM agar-slide for 28 h at 37 °C. Conidiophores were observed at the tip of aerial mycelium in wild type and the complemented strain. The Δ*AfusomA* mutant as well as the Δ*brlA* mutant showed only aerial hyphae on MM agar. Scale bars represent 20 μm (upper panel) and 50 μm (lower panel).

Asexual spores are produced at conidiophores consisting of aerial hyphae with a vesicle on top where the conidia are pinched off (Adams *et al.*, 1998). The Δ*AfusomA* strain as well as Δ*brlA* mutant formed exclusively aerial hyphae and was incapable to form conidiophores. To verify whether Δ*AfusomA* blocks

asexual development at early stage as the Δ*brlA* mutant, conidiophore morphology was examined under microscope. Wild type (AfS35), the Δ*AfusomA* mutant, the complemented strain and Δ*brlA* mutant were inoculated on MM-agar coated object-slide or MM agar on object-slide and incubated for 28 h at 37 °C. As shown in Figure 20B, microscopic inspection demenstrated that Δ*AfusomA* mutant showed no mature conidiophore formation similar to Δ*brlA* mutant. In contrast, the wild type AfS35 and the complemented strain revealed conidiophores (white arrow) and vesicle formation (black arrow) on top of the aerial hyphae. Furthermore, macroscopic inspection indicated that wild type produces aerial hyphae as well as conidiophores on the surface of a MM agar similar to the complemented strain. In contrast, Δ*AfusomA* and Δ*brlA* mutant showed only aerial hyphae (Figure 20B).

3.2.2 Xylose dependent expression of *AfusomA* shows different phenotypes compared to Δ*AfusomA* mutant and complemented strain

The BrlA protein represents a central regulator of conidiation. Defects in the corresponding gene result in a *bristle* (*brl*) phenotype due to a developmental block of conidiophore formation at the stage of aerial hyphae. Similarly, the Δ*AfusomA* mutant showed only aerial hyphae as *brlA* null strain, therefore we analyzed the AfuSomA dependent step in asexual development in more detail. A strain was constructed that could conditionally express *AfusomA* gene by xylose. The promoter region (602 bp) of *AfusomA* was replaced by inducible xylose dependent promoter (Figure 21A). The fragment containing the xylose driven *AfusomA* gene was transformed into wild type (AfS35) and the resulting *Xyl-AfusomA* strain (AfGB78) was confirmed by Southern hybridization with a probe detecting the 859 bp (position 1~859) of the *AfusomA* gene. Genomic DNA of the wild type and the *Xyl-AfusomA* strain were digested with *Sph*I restriction enzyme and showed a signal at about 2.8 kb for the wild type strain and 3.8 kb for the *Xyl-AfusomA* mutant (AfGB78) (Figure 21B).

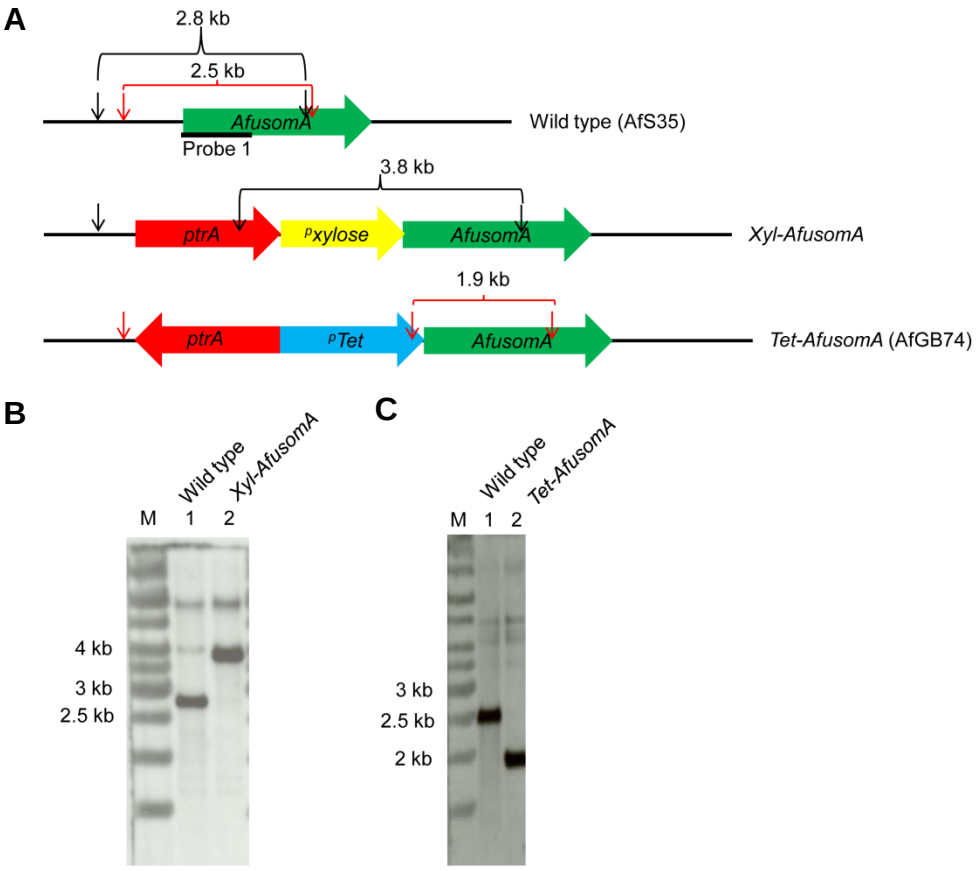


Figure 21. Scheme of two constructs for conditional expression of *AfusomA* and Southern hybridization.

(A) Wild type (AfS35) represents the original locus of *AfusomA* gene in chromosome. The follwing strains are the *Xyl-AfusomA* and the *Tet-AfusomA*. Green and red arrows represent *AfusomA* gene and pyrithiamine resistance (*prtA*) cassette. Xylose and tetracycline dependent promoters are shown with yellow and blue arrow, respectively. Probe used for Southern hybridization is indicated as horizontal black bar. Red and black vertical arrows indicate *Sac*I and *Sph*I restriction sites for DNA digestion, respectively. (B) Southern hybridization of wild type (Lane 1) and *Xyl-AfusomA* strain (lane 2). Genomic DNA was digested with *Sph*I restriction enzyme and 859 bp of *AfusomA* gene was used as probe for hybridization signal. The expected size of the wild type and the *Xyl-AfusomA* mutant is 2.8 kb and 3.8 kb. (C) Southern hybridization of wild type (Lane 1) and *Tet-AfusomA* strain (lane 2). Genomic DNA was digested with *Sac*I restriction enzyme and 859 bp of *AfusomA* gene was used as probe for hybridization signal. The expected size of the wild type and the *Tet-AfusomA* mutant is 2.5 kb and 1.9 kb.

The wild type (AfS35) and *Xyl-AfusomA* (AfGB78) strain were cultivated for five days on MM plates with (+) or without 1 % xylose. The wild type showed the same growth rate in the presence or absence of xylose. However, the color of conidiation was different in the plates supplemented with xylose (Figure 22), this indicated that conidiation of wild type might be affected by another carbon source. The *Xyl-AfusomA* mutant had severely impaired sporulation as Δ*AfusomA* mutant when xylose was absent (Off state), but the *Xyl-AfusomA* strain grew as fast as the complemented strain on MM plates without xylose. Furthermore, the conididaiton was partially restored in *Xyl-AfusomA* mutant in the presence of xylose (Figure 22).

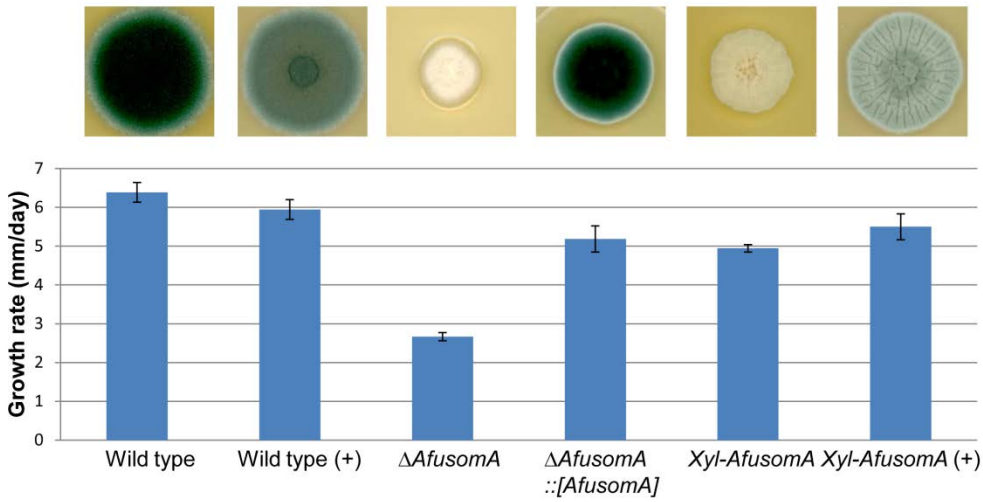


Figure 22. *Xyl-AfusomA* mutant shows a different phenotype compared to *AfusomA* null mutant and the wild type.

Colony morphology and growth rate of wild type (AfS35), Δ*AfusomA* mutant (AfGB72), the complemented strain Δ*AfusomA::[AfusomA]* (AfGB73) and *Xyl-AfusomA* mutant (AfGB78). All strains were grown on either MM plate (Off-state of the *Xyl*-promoter) or MM plate supplemented with 1 % xylose (On-state) for 5 days at 37 °C. Values in the graph are indicated as means ± standard error. Strains, grown on MM plate with 1 % xylose (On-state), are indicated with (+).

3.2.3 Doxycycline dependent expression of *AfusomA* shows a similar phenotype to Δ*AfusomA* mutant and the complemented strain

A different carbon source may have effects on the growth rate in *A. fumigatus* (Oliver *et al*., 2002). In addition, doxycycline dependent conditional expression systems have been used and improved in *A. fumigatus* (Helmschrott *et al*., 2013). Due to the fact that *Xyl-AfusomA* mutant revealed different phenotype to Δ*AfusomA* mutant and complemented strain, another strain was constructed using Tet-On expression system for *AfusomA* gene. Similar to *Xyl-AfusomA* strain, the promoter region (602 bp) of *AfusomA* was replaced by inducible Tet dependent promoter (Figure 21A). The fragment containing the Tet driven *AfusomA* gene was transformed into wild type (AfS35) and the resulting *Tet-AfusomA* strain (AfGB74) was confirmed by Southern hybridization with a probe detecting the *AfusomA* gene. Genomic DNA of the wild type and the *Tet-AfusomA* mutant were digested with *Sac*I restriction enzyme and had a signal at about 2.5 kb for the wild type strain and 1.9 kb for the *Tet-AfusomA* strain (Figure 21C). The wild type (AfS35) and *Tet-AfusomA* (AfGB74) strain were cultivated for five days on MM plates with or without the inducer. The wild type showed the same growth and conidiation phenotype in the presence or absence of doxycycline, which indicates that the additional doxycycline has not effects in wild type (Figure 23A). The *Tet-AfusomA* strain grew as slowly as the Δ*AfusomA* mutant and had severely impaired sporulation when doxycycline was absent (Off-state). In contrast, these impaired phenotypes were complemented when the promoter was induced by doxycycline (Figure 23A). Further observation showed that On-state of *Tet-AfusomA* strain revealed conidiophores (white arrow) and vesicle formation (black arrow) on top of the aerial hyphae as wild type; whereas the *Tet-AfusomA* strain in off state showed only aerial hyphae as Δ*AfusomA* mutant (Figure 23B).

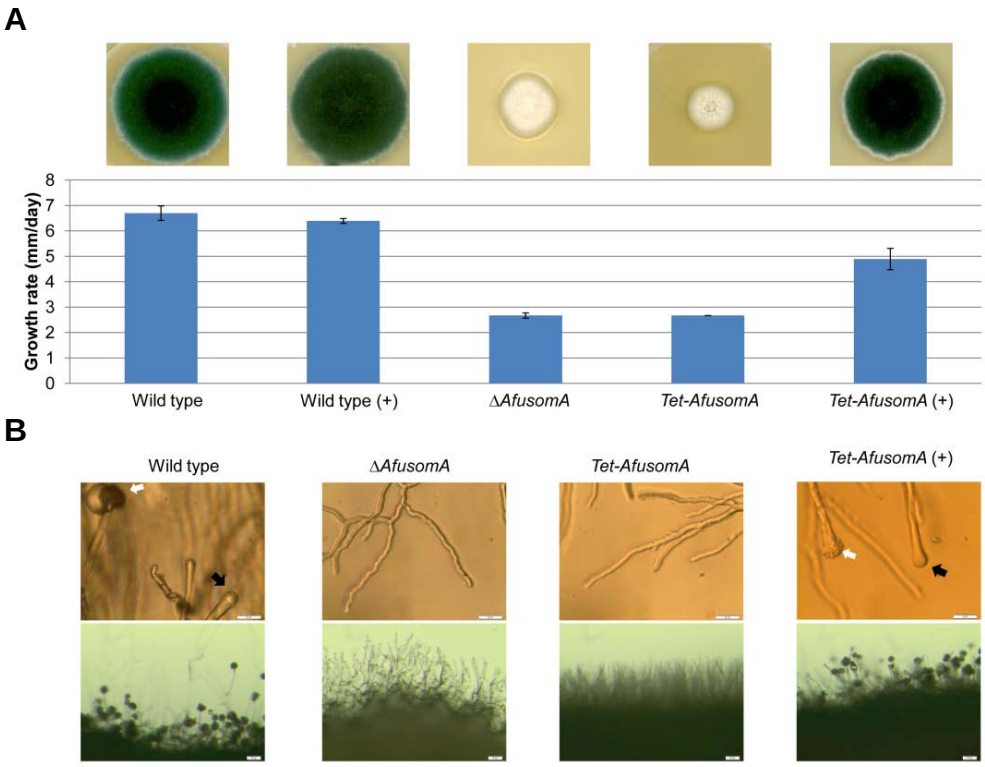


Figure 23. *Tet-AfusomA* mutant shows a similar phenotype like the Δ*AfusomA* mutant and the wild type.

(A) Colony morphology and growth rate of wild type (AfS35), Δ*AfusomA* mutant (AfGB72) and *Tet-AfusomA* strain (AfGB74). All strains were grown on either MM plate (Off-state of the *Tet*-promoter) or MM plate with 5 mg/l doxycycline (On-state) for 5 days at 37 °C. Values in the graph are indicated as means ± standard error. Strains, grown on MM plate with 5 mg/l doxycycline (On-state) are indicated with (+). (B) Morphology of conidiation in wild type, Δ*AfusomA* mutant and *Tet-AfusomA* strain. Upper panel: Strains were grown on MM or MM with doxycycline agar-coated slide for 28 h at 37 °C. In wild type and the *Tet-AfusomA* strain (On-state), white arrows indicate conidiophores and black arrows represent the vesicle for sporulation. Neither vesicles nor conidiophores were found in Δ*AfusomA* mutant and *Tet-AfusomA* strain without the inducer for *AfusomA* expression. Lower panel: Strains were grown on MM or MM with doxycycline agar-slide for 28 h at 37 °C. Conidiophores were observed at the tip of aerial mycelium in wild type and *Tet-AfusomA* strain in the On-state on MM containing doxycycline agar. Δ*AfusomA* mutant and *Tet-AfusomA* strain in the Off-state showed only aerial hyphae on MM agar, when the activating drug was absent. Scale bars represent 20 μm (upper panel) and 50 μm (lower panel). Strains, grown on MM plate with 5 mg/l doxycycline (On-state) are indicated with (+).

The combined data of the deletion analysis and the doxycycline dependent promoter suggest that AfuSomA is a regulator of asexual spore formation and is controlling developmental steps after the formation of aerial hyphae during the asexual cycle. The similar developmental phenotypes between the Δ*AfusomA* (AfGB72) and the Δ*brlA* (A1176) strain suggest that AfuSomA and BrlA might be part of the same regulatory pathway.

3.2.4 AfuSomA controls the expression of conidiation genes in *Aspergillus fumigatus*

The AfuSomA cellular function as transcription factor involved in asexual sporulation was examined by transcript analysis of putative target genes. Expression of *brlA* marks an initial step in asexual development where BrlA master transcription factor is synthesized (Yu, 2010). The Δ*AfusomA* strain showed a similar colony phenotype as the Δ*brlA* mutant strain (Figure 20). This suggests that AfuSomA regulates the expression of *brlA* gene or its upstream regulatory genes. qRT-PCR was performed with cDNA from wild type, Δ*AfusomA* mutant and the complemented strain, which were grown for 20 h at 37 °C. Transcript analysis revealed that the Δ*AfusomA* mutant strain abolished *brlA* expression in contrast to the wild type (AfS35) or the complemented strain. In *A. nidulans*, the *flbB*, *flbC*, and *flbD* genes encode regulators which are upstream of the activation of *brlA* expression. Further, FlbB is required for *flbD* expression (Garzia *et al.*, 2010; Kwon *et al.*, 2010a). Similar to *A. nidulans*, FlbB is necessary for *flbD* expression and FlbD might be essential for expression of *brlA* in *A. fumigatus* (Yu, 2010). The expression of *flbB* and *flbD* was decreased in the Δ*AfusomA* mutant whereas *flbC* expression was similar to the wild type control (Figure 24).

Apart from the upstream regulatory protein, the two transcription factors MedA and StuA are also required for conidiation in both *A. nidulans* and *A. fumigatus*. Loss of either *medA* or *stuA* results in abnormal conididation (Adams

et al., 1998; Sheppard *et al.*, 2005; Gravelat *et al.*, 2010). The transcript levels of *medA* and *stuA* were significantly reduced in the Δ*AfusomA* strain suggesting that AfuSomA affects the expression of *medA* and *stuA.* Further, the velvet domain protein family and the non-velvet protein and master regulator of secondary metabolism LaeA, which are conserved in ascomycetes and basidiomycetes (Ni and Yu, 2007), also control fungal development and secondary metabolism in filamentous fungi including conidiation (Bayram and Braus, 2012; Park *et al.*, 2012; Ahmed *et al.*, 2013). The expressions of members of the velvet domain protein family was not significantly affected except for transcription of the *velC* gene which was impaired by the *AfusomA* deletion mutant (Figure 24).

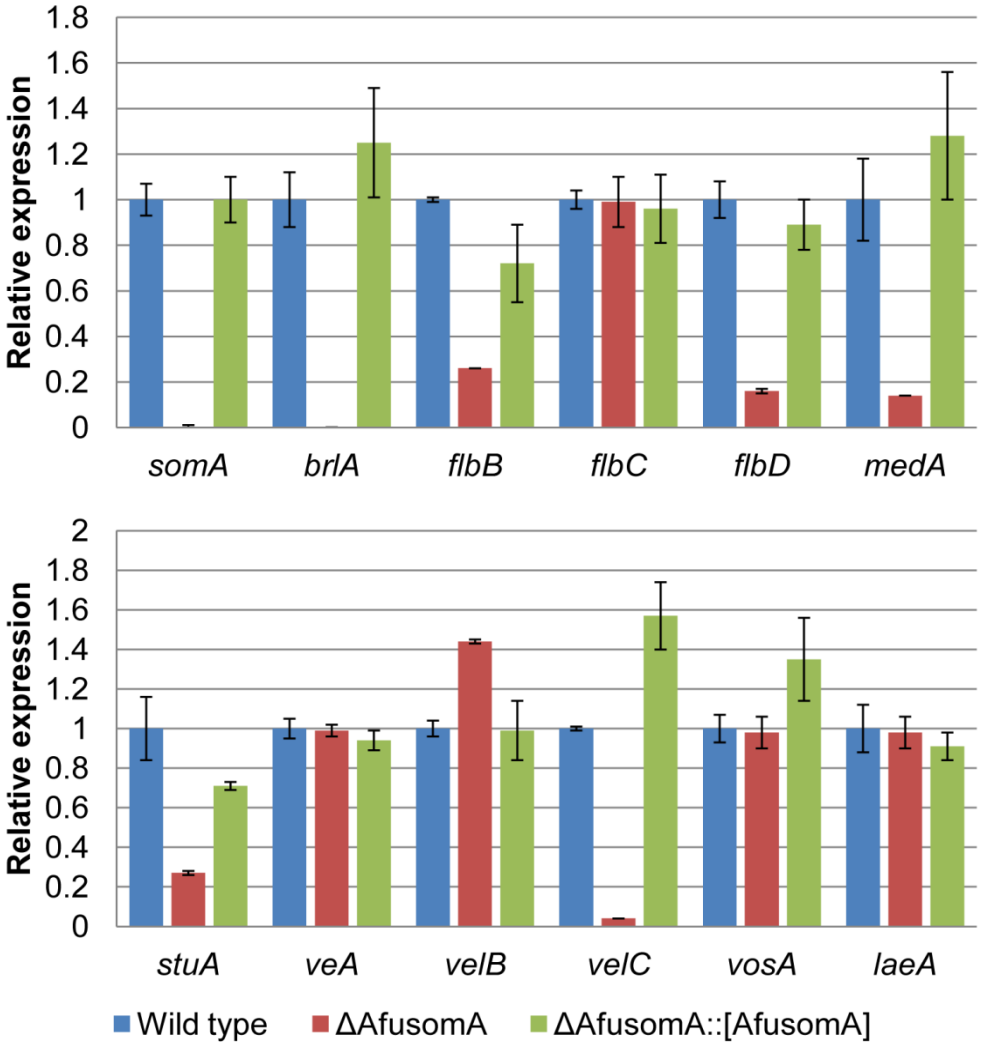


Figure 24. AfuSomA regulates genes for asexual development.

Relative expression of genes encoding proteins that regulate conidiation and adhesion, and *AfusomA* gene determined by quantitative RT-PCR. The *flbB*, *flbC* and *flbD* are upstream regulatory genes and *brlA* is the first gene for sporulation. The *vosA*,*veA*, *velB* and *velC* are velvet genes that play a role in fungal development. The *laeA* is regulator for development. The *medA* and *stuA* encode two transcriptional factors which regulate conidiation. Graph indicates mean ± standard errors and experiments were repeated for two times.

These data suggest that AfuSomA controls an entire network of conidiaton specific genes including *flbB* and *flbD* as early regulatory genes of asexual spore formation and *medA* and *stuA*, which are developmental modifier for spatial and temporal regulation of *brlA* expression .

3.2.5 AfuSomA interacts with PtaB in *Aspergillus fumigatus*

Flo8/Som1 proteins have been shown to interact with various proteins including Mss11, Mfg1, catalytic subunits of PKA and transcription factors of the APSES proteins (Yan *et al.*, 2011; McDonough and Rodriguez, 2012; Shapiro *et al.*, 2012). These interactions between Flo8/Som1 proteins with its' partner are required for regulating morphological development. We showed that AfuSomA activates *ScFLO11* transcription in ∆*flo8 S. cerevisiae* (Figure 17). This result suggested that AfuSomA might be regulated by catalytic subunits of PKA and interact with Mss11 and Mfg1 in yeast. To identify whether AfuSomA interacts with the homologs of Mfg1 or catalytic subunits of PKA in *A. fumigatus*, a GFP-Trap was performed with AfuSomA-sGFP tagged strain (AfGB75). The peptides from GFP-Trap were analyzed by LC-MS/MS. Data were collected at the highest expression levels of AfuSomA-GFP (24 h) from two independent samples from wild type (AfS35) expression sGFP protein as control and two independent AfuSomA-sGFP tagged strains. Proteins identified in sGFP control or only identified in one of AfuSomA-sGFP tagged strains were not included. All proteins that identified in GFP-Trap with AfuSomA are listed in Table 4. The protein name, gene number and function are according to the AspGD (http://www.aspergillusgenomes.org) (Arnaud *et al.*, 2012). Yeast homolog is according to the SGD (http://www.yeastgenome.org/).

Table 4. Proteins identified in the GFP-Trap.

Gene number	Protein name	Function	Yeast homolog	Reference
Afu7g02260	cAMP/PKA pathway (SomA)	Unknown	Flo8	
Afu2g12910	PtaB, putative	Unknown	Mfg1	(Conlon *et al.*, 2001)
Afu4g10460	Homocitrate synthase (HcsA)	Homocitrate synthase activity	Lys21	(Schobel *et al.*, 2010)
Afu1g14220	Nucleolar protein (NopA)	rRNA methyltransferase activity[1]	Nop1	(Tollervey *et al.*, 1991)
Afu3g09600	Pre-rRNA processing nucleolar protein Sik1, putative	Unknown	Nop56	
Afu4g09520	SNARE protein Ykt6, putative	Palmitoyltrasferase activity[1]	Ykt6	(Kweon *et al.*, 2003)
Afu3g07830	T-complex protein 1, delta subunit, putative	Unfolded protein binding[1]	Cct4	(Vinh and Drubin, 1994)
Afu3g13400	Putative nucleolar protein (Nop5)	Unknown	Nop58	
Afu4g09740	T-complex protein 1, theta subunit, putative	Unfolded protein binding[1]	Cct8	(Stoldt *et al.*, 1996)
Afu8g04730	Oligopeptidase family protein	Serine-type peptidase[2]	Ste13	(Hunter *et al.*, 2012)
Afu6g12740	Dienelactone hydrolase family protein	Hydrolase activity[2]	Aim2	(Hunter *et al.*, 2012)
Afu3g08380	Inorganic diphosphatase, putative (Ipp1)	Inorganic diphosphatase activity[1]	Ipp1	(Abadio *et al.*, 2011)

1: The function is according to the homologs in yeast; 2: The function of proteins is based on the conserved domain.

Twelve proteins including AfuSomA itself were identified that appear in both biological replicates, so very likely to interact with AfuSomA in *A. fumigatus* (Table 4). Two proteins (4g10460 and 3g08380) were also found in an independent GFP-Trap experiment using a different protein and might be considered as less likely for specific interaction. AfuPtaB (putative transcription activator) containing a LIM binding domain has a homolog in yeast called Mfg1 (Conlon *et al.*, 2001). It was identified as an interaction partner of Flo8 and Mss11 in both *S. cerevisiae* and *C. albicans* and this trimeric complex is required for activation of downstream targets (Ryan *et al.*, 2012). This interaction suggests that a AfuSomA-AfuPtaB complex might be conserved in fungi and plays a role to regulate gene expressions in *A. fumigatus.* The other interaction partners from the list have not been described to be involved in interaction of Flo8 in yeast. Several proteins are putatively ribosome and Golgi apparatus associated or have protein folding activity. The binding of these proteins might be unspecific and further investigations have to be carried out.

3.3 AfuSomA plays an important role in adhesion and virulence

3.3.1 AfuSomA is required for adherence to plastic surfaces

ScFlo8 as well as CaFlo8 regulates *FLO* genes or *ALS* genes encoding the corresponding adhesins in *S. cerevisiae* and *C. albicans*, respectively. Bioinformatic analysis predicted more than 100 proteins as putative adhesins in *A. fumigatus* (Upadhyay *et al.*, 2009; Chaudhuri *et al.*, 2011). Since AfuSomA could complement adhesion and activate *ScFLO11* expression in Δ*flo8* yeast mutant, we examined whether AfuSomA regulates adhesion in *A. fumigatus.* We compared adherence to plastic surfaces between the *A. fumigatus* wild type strain (AfS35) and the conditional *Tet-AfusomA* strain (AfGB74) where the promoter was either induced or shut off. As shown in Figure 25A, the wild type adhered strongly to plastic surfaces and this adhesion was not affected by the presence of doxycycline. The *Tet-AfusomA* strain resulted in similar adhesion as

wild type when the promoter was induced in MM with doxycycline. In contrast complete mycelium was washed off when the *Tet-AfusomA* strain was switched off in MM medium without the drug. This indicates that AfuSomA is part of the control for adherence to plastic in *A. fumigatus*.

The Flo8/Som1 homologs have been shown to interact with proteins which belong to APSES family such as StuA, and function downstream of the cAMP/PKA signaling pathway (Harcus *et al.*, 2004; Yan *et al.*, 2011). This interaction regulates the expression of adhesin genes (Cao *et al.*, 2006). The expression of *medA* and *stuA* had been shown to be regulated by AfuSomA (Figure 24). Gravelat *et al.* (2013) showed that the MedA and StuA proteins are required for adhesion and have also been shown to regulate putative adhesins by micro array and RT-PCR (Gravelat *et al.*, 2013). Therefore, three genes (*3g13110*, *3g00880* and *uge3*) encoding possible adherence-associated proteins with high scores in bioinformatic prediction (Chaudhuri *et al.*, 2011) were tested. The transcript levels of all three genes were reduced in the absence of AfuSomA (Figure 25B). This data suggests that AfuSomA controls StuA and MedA which regulate the expression of adhesins for adherence to plastic.

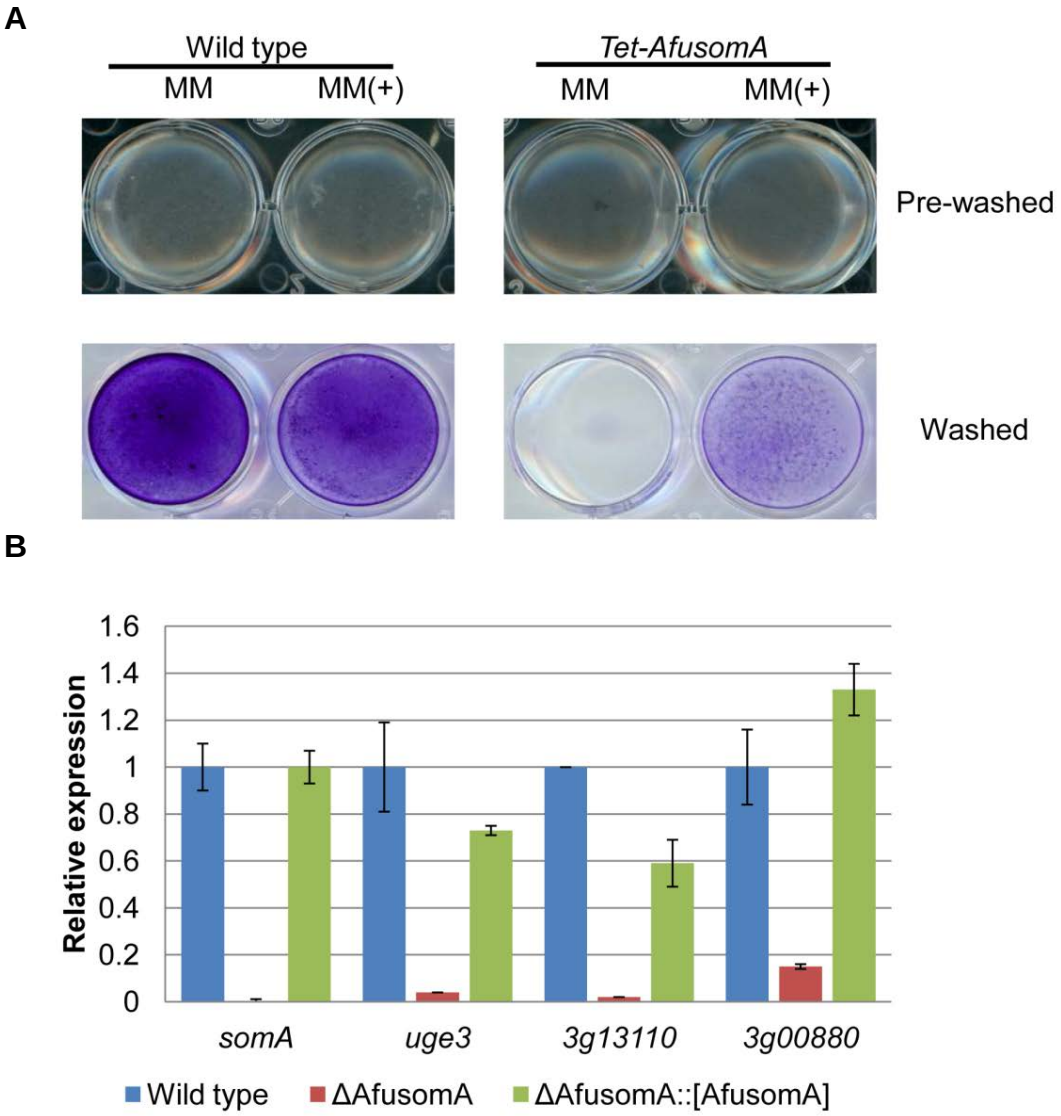


Figure 25. AfuSomA is required for adherence to plastic surfaces and regulates expression of putative adhesins.

(A) Adhesion assay of wild type and *Tet-AfusomA* mutant. Strains were grown in MM medium or MM with 5 mg/l doxycycline for 24 h. The wells were washed with PBS three times and mycelium was visualized by staining with 0.01 % crystal violet. Strains, grown in MM with 5 mg/l doxycycline are indicated with (+). (B) Relative expression of genes encoding proteins that regulate adhesion and *AfusomA* gene determined by quantitavie RT-PCR. The *uge3*, *3g13110* and *3g00800* are genes for UDP-Glucose epimerase and putative adhesins. Graph indicates mean ± standard errors and experiments were repeated two times.

3.3.2 AfuSomA is required for virulence in *Aspergillus fumigatus*

MedA is involved in adhesion of *A. fumigatus* and Δ*medA* results in reduced virulence in mice model (Gravelat *et al.*, 2010). We could show that AfuSomA is required for adhesion as well and also affects *medA* expression. Therefore we addressed whether AfuSomA plays a role in virulence. We compared the virulence of the wild type, the complemented strain and the *Tet-AfusomA* strain (On- and Off-state) in an egg infection model. The Δ*AfusomA* mutant was not included due to the severely impaired conidiation. This model is similar to the pulmonary invasive aspergillosis model in mice by infecting eggs in the chorioallantoic membrane (CAM) (Jacobsen *et al.*, 2010). The eggs infected with the inactive *Tet-AfusomA* strain without doxycycline had no significant difference compared to the PBS control. In contrast, the On state of the *Tet-AfuSomA* strain which was induced by doxycycline, showed similar virulence to wild type or the complemented strain (Figure 26).

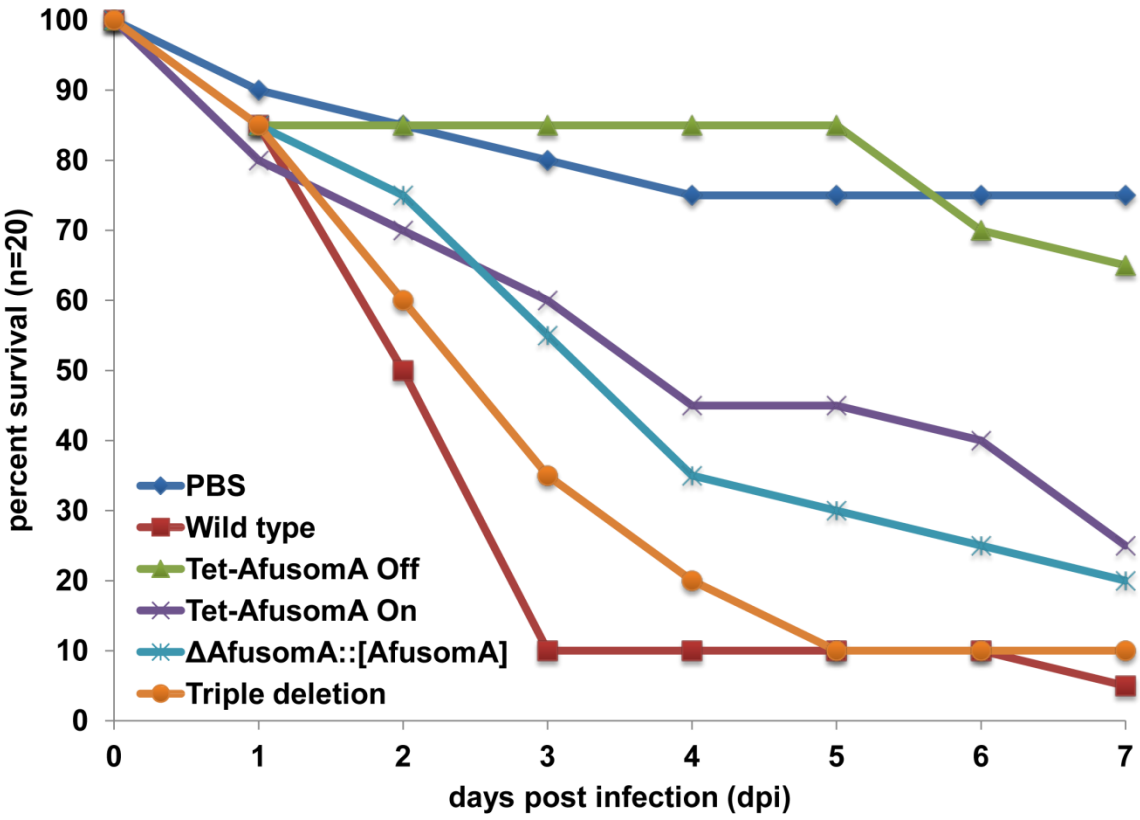


Figure 26. AfuSomA is required for virulence in an egg model of invasive aspergillosis.

20 eggs per strain were inoculated with 1000 conidia in 100 µl PBS with 5 mg/l doxycycline. Conidia from wild type (AfS35), the complemented strain Δ*AfusomA::[AfusomA]* (AfGB73), the *Tet-AfusomA* strain (AfGB74) and triple deletion of putative adhesins mutant (Δ*3g13110::*Δ*3g00880::*Δ*4g04070*) were used to perform the egg infection. No conidia could be used from Δ*AfusomA* mutant. Addition of doxycycline was not performed in the complemented, *Tet-AfusomA* Off state and triple deletion mutant. PBS control, wild type, *Tet-AfusomA* Off (silenced), *Tet-AfusomA* On (expressed), the complemented strain and triple deletion strain are shown in dark blue, red, green, purple, light blue and orange, respectively.

These data suggest that AfuSomA is contributing to virulence of the opportunistic fungal pathogen *A. fumigatus* in the egg model. Virulence features might be provided by the AfuSomA control of *medA* and *stuA*, which are required to promote the expression of adherence mediating genes like *uge3.* Consistently, the uridine diphosphate-glucose-epimerase Uge3, which is essential for

adherence through mediating the synthesis of galactosaminogalactan, has been shown to be essential for virulence in mice disease models (Gravelat *et al.*, 2013).

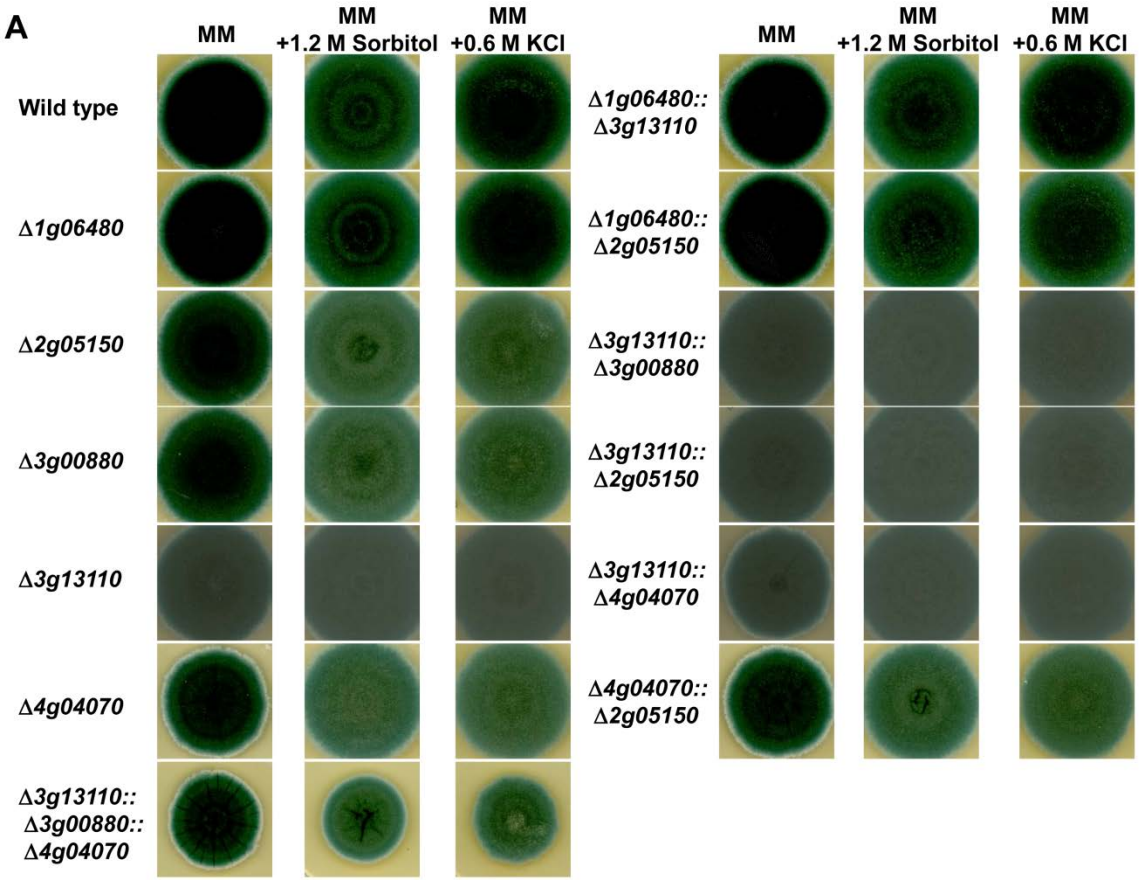
3.3.3 Triple deletion of putative adhesins has no effect on adhesion and virulence

AfuSomA has been shown to regulate expression of putative adhesins including *uge3, 3g00880* and *3g13110*. Disruption of *uge3* results in abolishment of adherence to plastic and human cells and reduced virulence in mice model of invasive aspergillosis (Gravelat *et al.*, 2013). Therefore, five putative adhesins (*1g06480*, *2g05150*, *3g00880*, *3g13110* and *4g04070*), which have a high score in bioinformatic prediction (Upadhyay *et al.*, 2009; Ramana and Gupta, 2010; Chaudhuri *et al.*, 2011), were deleted and were examined whether these putative adhesins plays a role in adherence to plastic. Single deletion mutants of each adhesin were attempted to be generated using the recyclable marker system. The mutants were constructed by transforming the corresponding deletion fragment, which was isolated from plasmids pME4200 to pME4204, into wild type strain (AfS35) to yield Δ*1g06480* (AfGB79), Δ*2g05150* (AfGB80), Δ*3g00880* (AfGB81), Δ*3g13110* (AfGB82) and Δ*4g04070* (AfGB83). To construct double or triple deletion mutants, the corresponding deletion fragment were transfored into the single deletion strain where the selective marker was removed, resulting Δ*1g06480::*Δ*2g05150* (AfGB84), Δ*1g06480::*Δ*3g13110* (AfGB85), Δ*3g13110::*Δ*2g05150* (AfGB86), Δ*3g13110::*Δ*3g00880* (AfGB87), Δ*3g13110::*Δ*4g04070* (AfGB88), Δ*4g04070::*Δ*2g05150* (AfGB89) and Δ*3g13110::*Δ*3g00880::*Δ*4g04070* (AfGB90). Southern hybridization was used to verify all of the deletion strains. for multiple deletion. To verified the *1g06480* deletion mutants, 5’ flanking region of *1g06480* as probe was used. Genomic DNA of wild type, Δ*1g06480::ptrA* and Δ*1g06480::six* strains was digested with *Nco*I restriction enzyme and showed a signal at about 4 kb for the wild type strain, 5 kb for the Δ*1g06480::ptrA* mutant and 2.2 kb for the Δ*1g06480::six* mutant (AfGB79). Similarly, a probe detecting 5’ flanking region of the *2g05150* gene

was used to examine the deletion mutants. Genomic DNA of wild type, Δ*2g05150::ptrA* and Δ*2g05150::six* strains were digested with *Sph*I restriction enzyme and showed a signal at about 3.3 kb for the wild type strain, 5.3 kb for the Δ*2g05150::ptrA* strain and 2 kb for the Δ*2g05150::six* mutant (AfGB80).The same procedure was applied to test the Δ*3g00880*, Δ*3g13110* and Δ*4g04070* mutants. To verify the Δ*3g00880* mutants, genomic DNA was digested with *Eco*RV restriction enzyme. The 5' flanking region was used as a probe and showed a signal at about 4.5 kb for the wild type strain, 8.5 kb for the Δ*3g00880::ptrA* mutant and 3.7 kb for the Δ*3g00880::six* mutant (AfGB81). To examine the Δ*3g13110* strains, 5' flanking region was used as a probe. Genomic DNA of wild type, Δ*3g13110::ptrA* and Δ*3g13110::six* strains were digested with *Xmn*I restriction enzyme and showed a signal at about 6.8 kb for the wild type strain, 3.3 kb for the Δ*3g13110::ptrA* and 2.7 kb for the Δ*3g13110::six* mutant (AfGB82). For Δ*4g04070* mutants, genomic DNA of wild type, Δ*4g04070::ptrA* and Δ*4g04070::six* strains were digested with *Pvu*II restriction enzyme. The 5' flanking region was used as a probe and showed a signal at about 2.3 kb for the wild type strain, 1.4 kb for the Δ*4g04070::ptrA* mutant and 2.7 kb for the Δ*4g04070::six* strain (AfGB83).

To test whether these putative adhesins play a role in cell wall integrity, growth test of the single, double and triple deletion mutants was performed. The MM plates and high osmolarity plates (MM with 1.2 M sorbitol or MM with 0.6 M KCl) were used to test these deletion mutants. Single adhesin deletion showed a similar phenotype compared to wild type as well as double deletion mutants. The triple deletion mutant (*3g13110::*Δ*3g00880::*Δ*4g04070*) revealed a slow growth rate on MM plates and on high osmolarity plates in comparison to wild type (Figure 27). Adhesins have been shown to be required for adhesion and virulence (de Groot *et al.*, 2013). We compared adherence to plastic surfaces between the wild type and the deletion mutants. The deletion mutants showed similar adhesion to wild type (Figure 28). Further analysis in egg model indicated that triple deletion mutant have no effect on virulence (Figure 26). This data

suggested that these putative adhesins contribute neither adherence to plastic surfaces nor virulence.



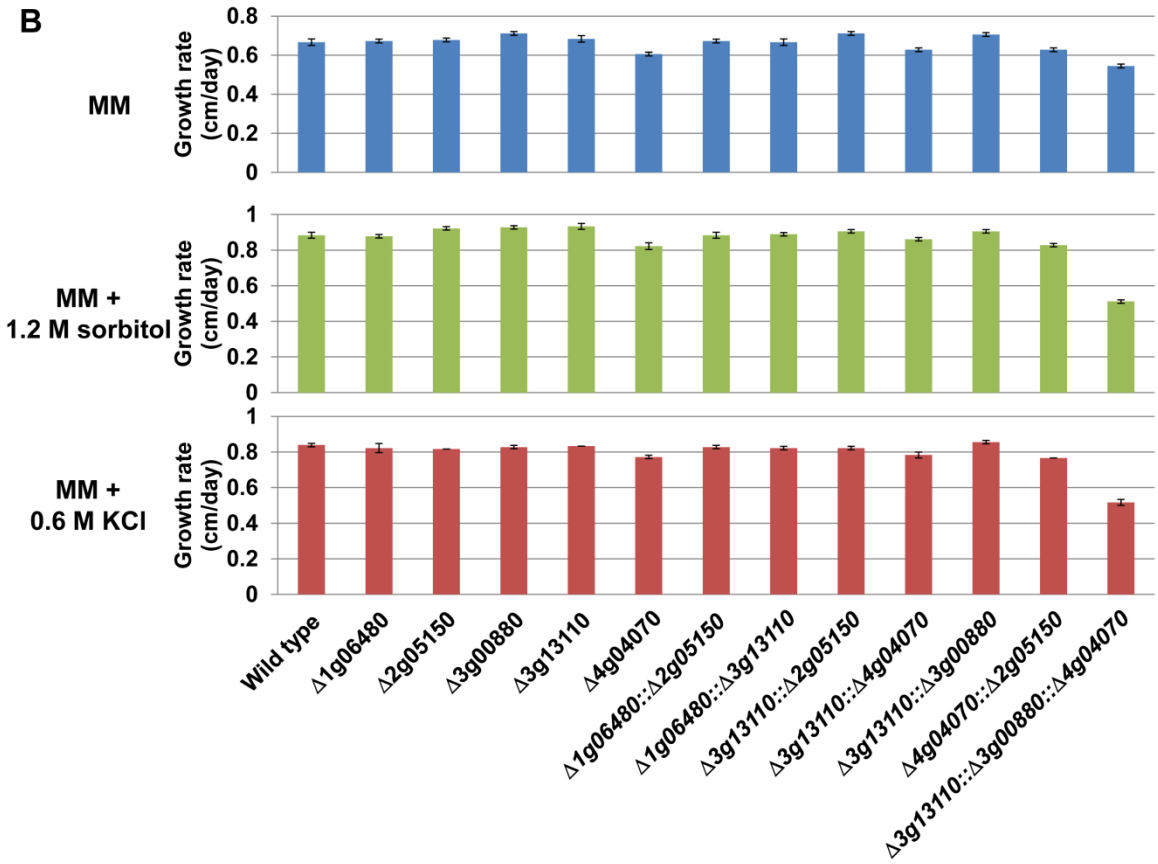


Figure 27. Triple adhesins deletion mutant shows reduced growth rate in *Aspergillus fumigatus*.

(A) Colony morphology and (B) growth rate of wild type (AfS35), single and multiple adhesins deletion mutants (Δ*1g06480*, Δ*2g05150*, Δ*3g00880*, Δ*3g13110*, Δ*4g04070*, Δ*1g06480::*Δ*3g13110*, Δ*1g06480::*Δ*2g05150*, Δ*3g13110::*Δ*3g00880*, Δ*3g13110::*Δ*2g05150*, Δ*3g13110::*Δ*4g04070*, *3g13110::*Δ*3g00880::*Δ*4g04070*). All strains were grown on MM plate and MM plate with either 1.2 M sorbitol or 0.6 M KCl for 5 days at 37 °C. Values in the graph are indicated as means ± standard error.

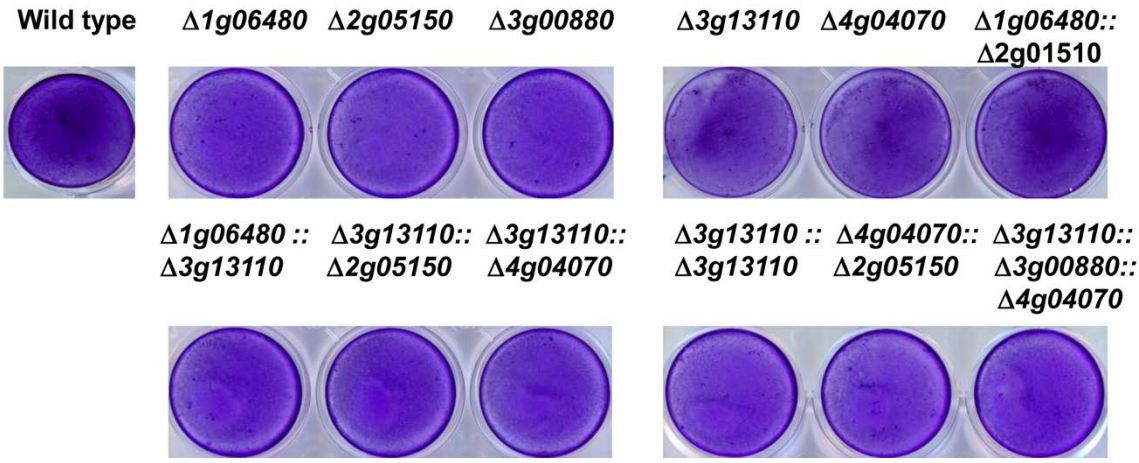


Figure 28. Deletion of putative adhesins has no effect on adhesion.

Adhesion assay for wild type (AfS35), single and multiple adhesins deletion mutants (Δ*1g06480*, Δ*2g05150*, Δ*3g00880*, Δ*3g13110*, Δ*4g04070*, Δ*1g06480::*Δ*3g13110*, Δ*1g06480::*Δ*2g05150*, Δ*3g13110::*Δ*3g00880*, Δ*3g13110::*Δ*2g05150*, Δ*3g13110::*Δ*4g04070*, *3g13110::*Δ*3g00880::*Δ*4g04070*). Strains were grown in MM for 24 h. The wells were washed with PBS three time and mycelium were visualized by staining with 0.01 % crystal violet.

4 Discussion

4.1 AfuSomA functions similar to ScFlo8 in *Saccharomyces cerevisiae*

4.1.1 Flo8/Som1 proteins are conserved in fungi

The cAMP/PKA pathway is conserved from fungi to mammals and regulates development and virulence in fungi (Lengeler *et al.*, 2000). The Flo8/Som1 transcription factors is one of various targets which functions downstream of cAMP/PKA pathway. The current understanding of Flo8/Som1 homologs and their role in adhesion and virulence is primarily based on yeasts with their dimorphic life style switching between a single cell yeast growth form and a pseudohyphal or hyphal growth mode (Liu *et al.*, 1996; Cao *et al.*, 2006). In addition, Som1 had been analyzed in plant pathogenic and saprophytic filamentous fungi that is responsible for development (Lee *et al.*, 2005; Yan *et al.*, 2011).

The Flo8/Som1 proteins share a conversed LUFS and LisH domains at their N-terminus. The LisH domain is responsible for protein dimerization and tetramerization (Cerna and Wilson, 2005). In yeasts, Flo8 binds to Mss11 protein which also contains LisH domain and this complex is required for activation of downstream genes (Kim *et al.*, 2004; Su *et al.*, 2009). Apart from Flo8/Som1 proteins, the LUFS domain can be found in single-stranded DNA binding proteins (ssdp) in mammal and *Drosophila* or *Arabidopsis* LEUNIG protein. Ssdp binds to Ldb1/Chip proteins which contain nuclear localization signal (NLS) but have no LUFS domain. This interaction is required for correct localization to the nucleus and consequently for the normal wing development in *Drosophila* (van Meyel *et al.*, 2003). Similarly, LEUNIG regulates flower development in *Arabidopsis* by interacting with SEUSS which shares similarity with Ldb1/Chip proteins (Franks *et al.*, 2002). However, there are some differences between ssdp, LEUNIG and Flo8/Som1 proteins. Ssdp is rather small than Flo8/Som1 and contains proline, glycine and methione rich domain at C-terminus (van Meyel *et al.*, 2003). LEUNIG is similar to Flo8/Som1 in size but harbors WD repeats which regulate

protein-protein interaction at C-terminus (Neer *et al.*, 1994; Conner and Liu, 2000). In contrast, there is no known conserved domain at the carboxyl site in Flo8/Som1 proteins. Furthermore, sequence of the NLS is conserved in Som1 proteins from filamentous fungi (Figure 11) but not in ssdp and LEUNIG. Taken together, the proteins containing LUFS domain play a role in development, but Flo8/Som1 protein might be a specific protein in fungi.

4.1.2 AfuSomA regulates adhesion in yeast

The Flo8 is one of the most prominent yeast regulators of adhesion and it had been demonstrated that Flo8 functions downstream of the cAMP/PKA pathway (Pan and Heitman, 2002). The binding of Flo8 to target promoters is regulated in budding yeast by Tpk2 which is one of the catalytic subunits of PKA, and loss of either Flo8 or Tpk2 blocks pseudohyphal growth (Pan and Heitman, 1999). Signal transduction through the PKA pathway is also functional when the yeast *FLO8* gene is replaced by heterologous expression of *MoSOM1* from the filamentous plant pathogen *M. oryzae* (Yan *et al.*, 2011) or *CaFLO8* and *AfusomA* from the opportunistic pathogen *C. albicans* (Cao *et al*., 2006) and *A. fumigatus* as shown in this study. Heterologous CaFlo8, MoSom1 or AfuSomA protein complement the defects of Δ*flo8* yeast in haploid adhesive or diploid pseudohyphal filamentous growth. These developmental programs including adhesion and pseudohyphal or hyphal growth require specific target genes mediating cell-cell or cell-surface interactions as *ScFLO11* or *CaHWP1* (Cao *et al.*, 2006; Hogan and Sundstrom, 2009; Brückner and Mösch, 2011; de Groot *et al.*, 2013). Expression of *ScFLO11* is regulated by numerous activating and repressing transcription factors in addition to Flo8 with Tup1, Hac1, Gcn4, Sfl1, Tec1 or Ste12 as prominent examples (Brückner and Mösch, 2011; Herzog *et al.*, 2013). Heterologous AfuSomA activates the expression of *ScFLO11* by binding to similar regions on the promoter as ScFlo8. This data suggest that AfuSomA might be activated by PKA and interact with Mss11 in yeast that further supports a common molecular mechanism of gene activation between yeasts and

filamentous fungi.

4.2 AfuSomA regulates asexual development at early stage in *Aspergillus fumigatus*

4.2.1 AfuSomA is required for asexual development

Asexual development in *Aspergilli* is a morphological change, which is reminiscent to the dimorphic life style of yeasts. Aerial hyphae are formed which can differentiate into conidiophores, containing many single cell conidia with a single nucleus per cell. These asexual spores are released into the air for dispersal of the fungus (Adams *et al.*, 1998; Yu, 2010; Krijgsheld *et al.*, 2013). Loss of *AfusomA* in *A. fumigatus* resulted in severely impaired conidiation, which suggests a function in the switch from the hyphae to the conidiophores development. This is consistent with earlier findings where lack of conidiation was also observed in *M. oryzae* and *A. nidulans* when Som1 is inactivated (Lee *et al.*, 2005; Yan *et al.*, 2011).

The connection between Flo8/Som1 and the PKA pathway seems to be conserved between yeasts and filamentous fungi. The Δ*AfusomA* strain in *A. fumigatus* was reduced in its growth rate in comparison to the wild type and resembles the Δ*acyA* mutant phenotype, which is deficient in the adenylyl cyclase producing cAMP where growth was reduced and nearly no conidiation was observed (Liebmann *et al.*, 2003). Furthermore, CaFlo8 and MoSom1 interact with the Tpk2 and CpkA catalytic subunit of protein kinase A, respectively (Harcus *et al.*, 2004; Yan *et al.*, 2011). *A. fumigatus pkaC1 and pkaC2* encode two cAMP dependent PKA catalytic subunits. PkaC1 belongs to the class I PKAs similar to Tpk proteins of *S. cerevisiae*. Whereas PkaC2 is dispensable for conidiation, while PkaC1 is responsible for conidiation and vegetative growth (Fuller *et al.*, 2011). This suggests that AfuSomA controls asexual development in response to the protein kinase PkaC1.

There is an important interplay between conidiation and cell-cell adhesion.

The formation of aerial hyphae results in vesicles, which further differentiate by a polar budding process reminiscent to yeast (Gimeno and Fink, 1994; Adams *et al.*, 1998). In *A. nidulans*, this results primarily in the formation of metulae cells which are absent in *A. fumigatus* where directly after vesicles formation the phialides are formed. Vesicles produce the elongated metulae and phialide cells in a process similar to pseudohyphae formation in yeast which requires adhesins such as Flo11, which mediate cell-cell adhesion (Brückner and Mösch, 2011). StuA controls metulae and phialide differentiation in *A. nidulans* and consistently, Phd1 as homolog of StuA, governs pseudohyphal growth in *S. cerevisiae* (Gimeno and Fink, 1994). Asexual spore formation also requires AbaA, which is located downstream of BrlA in the developmental cascade and is responsible for phialides formation. The corresponding yeast homolog Tec1, which is required for pseudohyphae formation can be replaced by *A. nidulans* AbaA to repair the defect of Δ*tec1 S. cerevisiae* mutant strain (Gavrias *et al.*, 1996). This suggests that AfuSomA might also regulate phialide formation.

4.2.2 AfuSomA controls expression of conidiation genes in *Aspergillus fumigatus*

AfuSomA controls conidiation primarily by affecting the expression of the three regulatory genes *flbB*, *stuA* and *medA* and consequently regulates the major regulator *brlA* expression (Figure 29). FlbB is a bZIP transcription factor which controls together with the cMyb factor FlbD the expression of *brlA*. The resulting protein BrlA is a C_2H_2 zinc finger transcription factor which plays a key role in asexual development in the pathogen *A. fumigatus* and the model fungus *A. nidulans* (Twumasi-Boateng *et al.*, 2009; Tao and Yu, 2011). Deletions of either *flbB* or *flbD* result in fluffy phenotypes resembling the Δ*brlA* mutant strain in *A. nidulans* (Garzia *et al*., 2009; Garzia *et al*., 2010). The FlbB impact on conidiation is similar in *A. fumigatus*, but the FlbD impact is less pronounced. A *flbB* deletion abolishes *flbD* expression and delays *brlA* expression. In *A. fumigatus*, expression of the *flbD* gene requires in addition to FlbB also FlbE as

further developmental regulator. Consequently, conidiation is delayed and reduced in a Δ*flbB* mutant (Xiao *et al.*, 2010; Yu, 2010). However, conidiation is abolished in a Δ*AfusomA* mutant (Figure 23). This indicates that other developmental regulator might be required for normal conidiation.

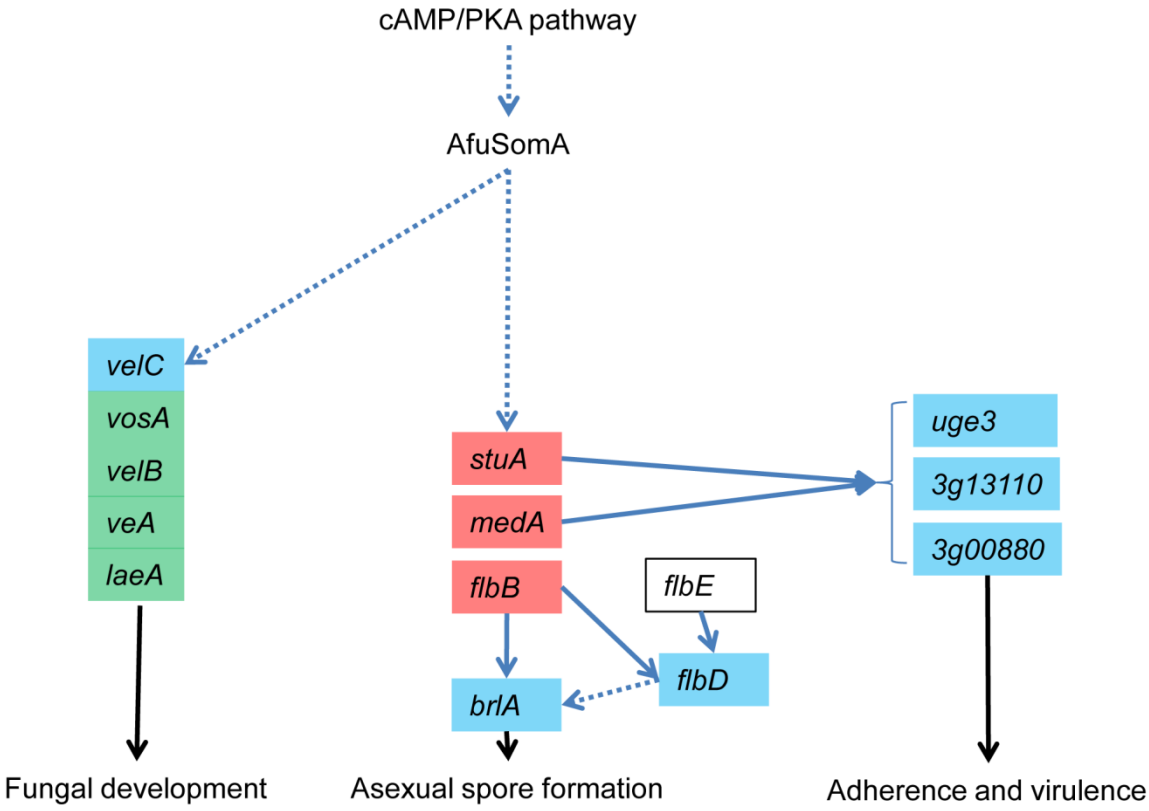


Figure 29. Model of AfuSomA controls conidiation, adhesion and virulence in *Aspergillus fumigatus*.

The model describes AfuSomA which might be activated by cAMP/PKA pathway activate the transcriptional network directly or indirectly. The *flbB*, *medA*, and *stuA* (red) are regulatory genes, which control conidiation and adherence. The *flbE* is regulator of *flbD*. Genes boxed in blue are presumably indirectly regulated by AfuSomA, whereas genes in green are not affected in their expression by AfuSomA. Solid blue arrows are further supported by previous studies (Garzia *et al.*, 2010; Kwon *et al.*, 2010b; Xiao *et al.*, 2010; Gravelat *et al.*, 2013).

AfuSomA controls expression of StuA and MedA encoding genes which regulate conidiation in *A. fumigatus*. StuA and MedA contribute to the proper spatial and temporal expression of *brlA.* Consistently, disruption of *stuA* and

medA result in abnormal conidiophores and reduced conidiation in *A. nidulans* and *A. fumigatus* (Adams *et al.*, 1998; Sheppard *et al.*, 2005; Gravelat *et al.*, 2010). The StuA binding sites (A/T)CGCG(T/A)N(A/C) had been defined (Dutton *et al.*, 1997) and are present in the *brlA* promoter region (position -507, -753 and -3276 bp) (Figure 30) for asexual development, while the MedA binding sites are still unclear. Taken together, the severe impairment of conidiation in the *AfusomA* deletion mutant can be attributed to FlbB, StuA and MedA (Figure 29).

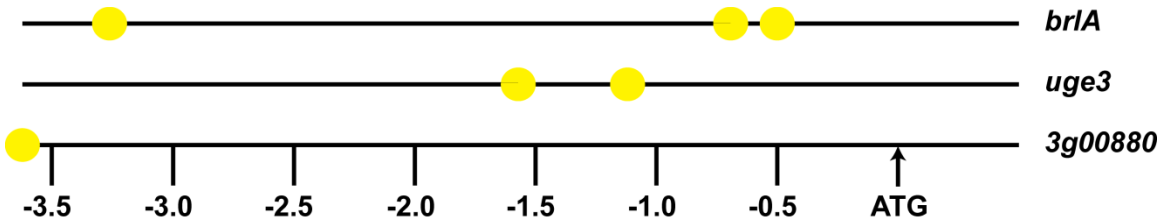


Figure 30. The StuA DNA binding sites in regulated genes.

Positions of StuA binding sites (A/T)CGCG(T/A)N(A/C) (Dutton *et al.*, 1997) in 3.5 kb promoter of *brlA, uge3* and *3g00880* genes.

The velvet protein family and LaeA have been shown to regulate development in *A. nidulans* and *A. fumigatus* (Bayram and Braus, 2012; Park *et al.*, 2012). However, only *velC* expression was regulated by AfuSomA (Figure 24). Recently, Park and his colleagues showed that VelC positively controls sexual development in *A. nidulans* (Park *et al.*, 2014). This suggest that AfuSomA might play a role in sexual development in *A. fumigatus*.

4.2.3 AfuSomA interacts with PtaB in *Aspergillus fumigatus*

Flo8/Som1 is a transcription factor that regulates downstream targets together with other interaction partners (Yan *et al.*, 2011; Ryan *et al.*, 2012). Here we showed that AfuSomA interacts with AfuPtaB the Mfg1 homolog which is a highly conversed protein in eukaryotic cells. In *S.* cerevisiae Mfg1 forms a

complex with Mss11 and Flo8 leading to efficient Flo11 expression in this organism and hence mediating invasive growth and pseudohyphal formation (Ryan *et al.*, 2012). Deletion of each partner leads to loss of this phenotype. Mfg1 is a conversed protein containing LIM-binding domain, a protein binding domain that is found in proteins that are involved in development and cytoskeleton organization in yeast, fly and human (van Meyel *et al.*, 2003; Koch *et al.*, 2012; Shapiro *et al.*, 2012). Although an interaction of AfuSomA occurs with AfuPtaB as shown in the GFP-trap, the situation is different in *A. fumigatus* compared to both yeast. Mss11, which contains like Flo8 a LisH domain to bind to each other has no homologous protein in filamentous fungi. Therefore three options are possible that occur in *A. fumigatus*: (1) an unidentified interaction partner overtake Mss11; (2) no additional binding protein is needed for function of adhesive and developmental growth; or (3) AfuSomA with the LisH domain binds twice to AfuPtaB, overtaking the function of Mss11 (Figure 31). Taken together, a heterocomplex of AfuSomA and AfuPtaB might play an important role in activation transcriptional network.

Figure 31. Model of AfuSomA and PtaB complex.

Three possible AfuSomA/PtaB complexes in *A. fumigatus*.

AfuSomA could complement the pseudohyphal growth and activate the *ScFLO11* expression in Δ*flo8* yeast. This indicates that AfuSomA interacts with catalytic subunits of PKA. However, neither PkaC1 nor PkaC2 were identified in mass spectrometry. Phosphorylation is required for ScFlo8 function in yeast, and is a transient action. MoSom1 has been shown to interact with CpkA catalytic subunits in the present of cAMP which liberates CpkA from its regulatory

subunits (Yan *et al*., 2011).

4.3 AfuSomA plays an important role in adhesion and virulence

Bacteria like *Streptococci* express different adhesins to bind to components of the extracellular matrix like collagen, fibrinogen, laminin or fibronectin (Rohde and Chhatwal, 2013). In *Streptococcus pyogenes*, the adhesins (M proteins) bind to plasminogen and plasmin. In addition, they also mediate invasion as the important step in the infection process and the resulting pathogenesis (Siemens *et al.*, 2011). Adhesion to abiotic surfaces of medical devices can result in the formation of biofilms. This is a serious problem because it facilitates infection by the dimorphic ascomycetous yeast *C. albicans* as human pathogen (Uppuluri and Lopez-Ribot, 2010; Mayer *et al.*, 2013). Adhesion and virulence have also been shown to be interdependent for the cell flocculin 1 of the dimorphic basidomycete yeast *C. neoformans* or the spherule outer wall glycoprotein of *Coccidioides immitis* which causes primary mycoses (de Groot *et al.*, 2013).

4.3.1 AfuSomA is required for adherence and virulence in *Aspergillus fumigatus*

Two transcription factors, StuA and MedA, have been shown to regulate not only conidiation but also adherence in *A. fumigatus* (Sheppard *et al.*, 2005; Gravelat *et al.*, 2013). Uge3 is a UDP-glucose epimerase that interconverts UDP-glucose and UDP galactose and mediates formation of galactosaminogalactan. This compound is part of the extracellular matrix and is required for biofilm formation as well as adherence and therefore plays a prominent role in pathogenesis of *A. fumigatus* (Loussert *et al.*, 2010). Deletion of *stuA* or *medA* results in no biofilm formation and these two gene are required for *uge3* expresion. Disruption of *uge3* in *A. fumigatus* shows reduced adherence to

plastic and attenuated virulence in mice model of invasive aspergillosis (Gravelat *et al.*, 2013). Conditional expression *AfusomA* mutant showed no adherence to plastic and reduced virulence in egg model of invasive aspergillosis when *AfusomA* is inactivated. Further, the StuA binding sites are also present in the promoter regions of the *uge3* (position -1651 and -1108 bp) or *3g00880* (position -3627 bp) genes for adhesion. This indicated that StuA has the dual role in directly activating the transcription of genes for adhesion and conidiation by binding to the corresponding promoters (Figure 29). Taken together, AfuSomA is required for the expression of *stuA* and *medA* and thereby plays an important role for adhesion as well as virulence.

4.3.2 Putative adhesins are not required for either adhesion or virulence

Several proteins which provide adherence in filamentous fungi have been identified. Galactosaminogalactan is known to be an adhesive compound produced by the *A. fumigatus* Uge3 epimerase, which is under AfuSomA control. Hydrophobin Mpg1 in plnt pathogen *Magnaporthe grisea* is responsible for appressorium development and subsequent entry into the plant host (Talbot *et al.*, 1993). RodA is a spore hydrophobin of *A. fumigatus* which provides adherence of conidia to collagen or albumin (Thau *et al.*, 1994). The expression of the *rodA* gene depends on regulators as BrlA and AbaA (Yu, 2010). Therefore the *rodA* expression is probably affected by AfuSomA, since *brlA* expression is repressed in Δ*AfusomA* mutant.

Although proteins mentioned above play a role in adherence to different surfaces, but the adhesins which is required for adhesion as *ScFLO11* or *CaALS1* are still not identified in *A. fumigatus*. Several bioinformatic tools have been developmed and used to identify putative adhesins containing GPI anchor and serine-threonine rich domain in *A. fumigatus* (Levdansky *et al.*, 2007; Upadhyay *et al.*, 2009; Ramana and Gupta, 2010; Chaudhuri *et al.*, 2011). Two adhesins (CalA and CspA) have been characterized and shown to be

responsible for adherence to laminin and extracellular matrix of alveolar epithielial cells. In this study, five putative adhesins were deleted and examined whether them contribute to adherence or virulence. In single, double and triple adhesins deletion mutants, normal adherence to plastic in comparison to wild type were observed. This negative result indicates that either bioinformatic prediction of adhesin does not fit in *A. fumigatus* or the redundant genes can take over the function of deleted genes (Hartmann *et al.*, 2011; Amich and Krappmann, 2012).

4.4 Outlook

In this study, we conclude that AfuSomA plays an important role in the transcriptional network that controls morphological development as well as adhesion which is important for pathogenesis in the opportunistic pathogen *A. fumigatus*. The molecular mechanism of asexual development in *Aspergilli* is well developed, especially how does the expression of *brlA* be activated (Krijgsheld *et al.*, 2013). We showed that AfuSomA regulates *brlA* expression. Recently, NsdD protein has been shown to be a key repressor of asexual development in *A. nidulans.* Deletion of NsdD overcome the need for upstream regulators such as *flbB* and *flbD* in conidiation, but does not complement Δ*brlA* phenotype (Lee et al., 2014). Due to the fact that *flbB* and *flbD* were regulated by AfuSomA in *A.* fumigatus. It would be interesting to examine whether the deletion of NsdD in Δ*AfusomA* mutant where has no *brlA* expression can restore the defect of conidiation.

The Flo8 forms a complex with Mss11 and Mfg1 in both *S. cerevisiae* and *C. albicans* to regulate morphological transition. Deletion of either gene abolishes the pseudohyphal or hyphal growth in yeasts (Shapiro *et al.*, 2012). In this study we showed that AfuSomA interacts with PtaB which is the homolog of Mfg1. In future studies, It will be essential to test whether PtaB plays a similar role with AfuSomA in development and adhesion. Furthermore, both AfuSomA and PtaB

harbor predicted NLS signal. It would be interesting to test that these two proteins enter nucleus before or after forming the complex. We presented that AfuSomA and ScFlo8 act on similar region of *FLO11* promoter in yeast. But the DNA binding motif of ScFlo8 does not present in the promoter of AfuSomA regulated genes. In order to know whether AfuSomA directly activates the transcription of conidiation genes. One possibility is electrophoretic mobility shift assay.

Adhesins play a role in virulence in *C. albicans* (de Groot *et al*., 2013). In this study we showed that five predicted adhesins had no significant role in adherence to plastic surface and virulence in egg model of invasive aspergillosis. *S. cerevisiae* has been used as a tool to identify control genes of adhesion from filamentous fungus *Verticillium longisporum* (Tran *et al*., 2014). However, no adhesins were identified from the screening. In future studies, it would be possible to identify the adhesins which can be regulated by StuA, AbaA and AfuSomA. Because the homologs of these proteins are required for pseudohyphal growth in *S. cerevisiae*. Furthermore, phialides formation which is similar to pseudohyphal formation might also needs adhesins to perform cell-cell adhesion.

5 Reference

Abad, A., Victoria Fernandez-Molina, J., Bikandi, J., Ramirez, A., Margareto, J., Sendino, J., Luis Hernando, F., Ponton, J., Garaizar, J., and Rementeria, A. (2010). What makes *Aspergillus fumigatus* a successful pathogen? Genes and molecules involved in invasive aspergillosis. Rev Iberoam Micol *27*, 155-182.

Abadio, A.K., Kioshima, E.S., Teixeira, M.M., Martins, N.F., Maigret, B., and Felipe, M.S. (2011). Comparative genomics allowed the identification of drug targets against human fungal pathogens. BMC Genomics *12*, 75.

Adams, T.H., Wieser, J.K., and Yu, J.H. (1998). Asexual sporulation in *Aspergillus nidulans*. Microbiol Mol Biol Rev *62*, 35-54.

Agarwal, C., Aulakh, K.B., Edelen, K., Cooper, M., Wallen, R.M., Adams, S., Schultz, D.J., and Perlin, M.H. (2013). *Ustilago maydis* phosphodiesterases play a role in the dimorphic switch and in pathogenicity. Microbiology *159*, 857-868.

Ahmed, Y.L., Gerke, J., Park, H.S., Bayram, Ö., Neumann, P., Ni, M., Dickmanns, A., Kim, S.C., Yu, J.H., Braus, G.H., and Ficner, R. (2013). The velvet family of fungal regulators contains a DNA-binding domain structurally similar to NF-κB. PLoS Biol *11*, e1001750.

Amich, J., and Krappmann, S. (2012). Deciphering metabolic traits of the fungal pathogen *Aspergillus fumigatus*: redundancy vs. essentiality. Front Microbiol *3*, 414.

Arnaud, M.B., Cerqueira, G.C., Inglis, D.O., Skrzypek, M.S., Binkley, J., Chibucos, M.C., Crabtree, J., Howarth, C., Orvis, J., Shah, P., Wymore, F., Binkley, G., Miyasato, S.R., Simison, M., Sherlock, G., and Wortman, J.R. (2012). The Aspergillus Genome Database (AspGD): recent developments in comprehensive multispecies curation, comparative genomics and community resources. Nucleic Acids Res *40*, D653-659.

Askew, D.S. (2008). *Aspergillus fumigatus*: virulence genes in a street-smart mold. Curr Opin Microbiol *11*, 331-337.

Bölker, M. (2001). *Ustilago maydis*--a valuable model system for the study of fungal dimorphism and virulence. Microbiology *147*, 1395-1401.

Bayram, Ö., and Braus, G.H. (2012). Coordination of secondary metabolism and development in fungi: the velvet family of regulatory proteins. FEMS Microbiol Rev *36*, 1-24.

Bayry, J., Aimanianda, V., Guijarro, J.I., Sunde, M., and Latgé, J.P. (2012). Hydrophobins—unique fungal proteins. PLoS Pathog *8*, e1002700.

Beauvais, A., Schmidt, C., Guadagnini, S., Roux, P., Perret, E., Henry, C., Paris, S., Mallet, A., Prevost, M.C., and Latgé, J.P. (2007). An extracellular matrix glues together the aerial-grown hyphae of *Aspergillus fumigatus*. Cell Microbiol *9*, 1588-1600.

Bertani, G. (1951). Studies on lysogenesis. I. The mode of phage liberation by lysogenic *Escherichia coli*. J Bacteriol *62*, 293-300.

Bester, M.C., Pretorius, I.S., and Bauer, F.F. (2006). The regulation of *Saccharomyces cerevisiae FLO* gene expression and Ca2+ -dependent flocculation by Flo8p and Mss11p. Curr Genet *49*, 375-383.

Blackwell, M. (2011). The fungi: 1, 2, 3 ... 5.1 million species? Am J Bot *98*, 426-438.

Boylan, M.T., Mirabito, P.M., Willett, C.E., Zimmerman, C.R., and Timberlake, W.E. (1987). Isolation and physical characterization of three essential conidiation genes from *Aspergillus nidulans*. Mol Cell Biol *7*, 3113-3118.

Brückner, S., and Mösch, H.U. (2011). Choosing the right lifestyle: adhesion and development in Saccharomyces cerevisiae. FEMS Microbiol Rev *36*, 25-58.

Bradford, M.M. (1976). A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Analytical biochemistry *72*, 248-254.

Braus, G.H., Grundmann, O., Brückner, S., and Mösch, H.U. (2003). Amino acid starvation and Gcn4p regulate adhesive growth and *FLO11* gene expression in *Saccharomyces cerevisiae*. Mol Biol Cell *14*, 4272-4284.

Cao, F., Lane, S., Raniga, P.P., Lu, Y., Zhou, Z., Ramon, K., Chen, J., and Liu, H. (2006). The Flo8 transcription factor is essential for hyphal development and virulence in *Candida albicans*. Mol Biol Cell *17*, 295-307.

Caracuel, Z., Roncero, M.I., Espeso, E.A., González-Verdejo, C.I., Garcia-Maceira, F.I., and Di Pietro, A. (2003). The pH signalling transcription factor PacC controls virulence in the plant pathogen *Fusarium oxysporum*. Mol Microbiol *48*, 765-779.

Carrion Sde, J., Leal, S.M., Jr., Ghannoum, M.A., Aimanianda, V., Latgé, J.P., and Pearlman, E. (2013). The RodA hydrophobin on *Aspergillus fumigatus* spores masks dectin-1- and dectin-2-dependent responses and enhances fungal survival in vivo. J Immunol *191*, 2581-2588.

Cerna, D., and Wilson, D.K. (2005). The structure of Sif2p, a WD repeat protein functioning in the SET3 corepressor complex. J Mol Biol *351*, 923-935.

Chang, M.H., Chae, K.S., Han, D.M., and Jahng, K.Y. (2004). The GanB Galpha-protein negatively regulates asexual sporulation and plays a positive role in conidial germination in *Aspergillus nidulans*. Genetics *167*, 1305-1315.

Chaudhuri, R., Alam Ansari, F., Raghunandanan, M.V., and Ramachandran, S. (2011). FungalRV: Adhesin prediction and immunoinformatics portal for human fungal pathogens. BMC Genomics *12*, 192-205.

Cherry, J.M., Hong, E.L., Amundsen, C., Balakrishnan, R., Binkley, G., Chan, E.T., Christie, K.R., Costanzo, M.C., Dwight, S.S., Engel, S.R., Fisk, D.G., Hirschman, J.E., Hitz, B.C., Karra, K., Krieger, C.J., Miyasato, S.R., Nash, R.S., Park, J., Skrzypek, M.S., Simison, M., Weng, S., and Wong, E.D. (2012). *Saccharomyces* Genome Database: the genomics resource of budding yeast. Nucleic Acids Res *40*, D700-705.

Conlon, H., Zadra, I., Haas, H., Arst, H.N., Jr., Jones, M.G., and Caddick, M.X. (2001). The *Aspergillus nidulans* GATA transcription factor gene *areB* encodes at least three proteins and features three classes of mutation. Mol Microbiol *40*, 361-375.

Conner, J., and Liu, Z. (2000). *LEUNIG*, a putative transcriptional corepressor that regulates *AGAMOUS* expression during flower development. Proc Natl Acad Sci U S A *97*, 12902-12907.

Cullen, P.J., and Sprague, G.F., Jr. (2000). Glucose depletion causes haploid invasive growth in yeast. Proc Natl Acad Sci U S A *97*, 13619-13624.

Cullen, P.J., and Sprague, G.F., Jr. (2012). The regulation of filamentous growth in yeast. Genetics *190*, 23-49.

Dagenais, T.R., and Keller, N.P. (2009). Pathogenesis of *Aspergillus fumigatus* in Invasive Aspergillosis. Clin Microbiol Rev *22*, 447-465.

de Groot, P.W., Bader, O., de Boer, A.D., Weig, M., and Chauhan, N. (2013). Adhesins in human fungal pathogens: glue with plenty of stick. Eukaryot Cell *12*, 470-481.

de Groot, P.W., Hellingwerf, K.J., and Klis, F.M. (2003). Genome-wide identification of fungal GPI proteins. Yeast *20*, 781-796.

de Groot, P.W., Kraneveld, E.A., Yin, Q.Y., Dekker, H.L., Groß, U., Crielaard, W., de Koster, C.G., Bader, O., Klis, F.M., and Weig, M. (2008). The cell wall of the human pathogen *Candida glabrata*: differential incorporation of novel adhesin-like wall proteins. Eukaryot Cell *7*, 1951-1964.

Di Pietro, A., Garcia-MacEira, F.I., Meglecz, E., and Roncero, M.I. (2001). A MAP kinase of the vascular wilt fungus *Fusarium oxysporum* is essential for root penetration and pathogenesis. Mol Microbiol *39*, 1140-1152.

Dorsam, R.T., and Gutkind, J.S. (2007). G-protein-coupled receptors and cancer. Nat Rev Cancer *7*, 79-94.

Dranginis, A.M., Rauceo, J.M., Coronado, J.E., and Lipke, P.N. (2007). A biochemical guide to yeast adhesins: glycoproteins for social and antisocial occasions. Microbiol Mol Biol Rev *71*, 282-294.

Dubey, M.K., Jensen, D.F., and Karlsson, M. (2014). Hydrophobins are required for conidial hydrophobicity and plant root colonization in the fungal biocontrol agent *Clonostachys rosea*. BMC Microbiol *14*, 18.

Durrenberger, F., Wong, K., and Kronstad, J.W. (1998). Identification of a cAMP-dependent protein kinase catalytic subunit required for virulence and morphogenesis in *Ustilago maydis*. Proc Natl Acad Sci U S A *95*, 5684-5689.

Dutton, J.R., Johns, S., and Miller, B.L. (1997). StuAp is a sequence-specific transcription factor that regulates developmental complexity in *Aspergillus nidulans*. EMBO J *16*, 5710-5721.

Etxebeste, O., Garzia, A., Espeso, E.A., and Ugalde, U. (2010). *Aspergillus nidulans* asexual development: making the most of cellular modules. Trends Microbiol *18*, 569-576.

Etxebeste, O., Ni, M., Garzia, A., Kwon, N.J., Fischer, R., Yu, J.H., Espeso, E.A., and Ugalde, U. (2008). Basic-zipper-type transcription factor FlbB controls asexual development in *Aspergillus nidulans*. Eukaryot Cell *7*, 38-48.

Fichtner, L., Schulze, F., and Braus, G.H. (2007). Differential Flo8p-dependent regulation of *FLO1* and *FLO11* for cell-cell and cell-substrate adherence of *S. cerevisiae* S288c. Mol Microbiol *66*, 1276-1289.

Filler, S.G., and Sheppard, D.C. (2006). Fungal invasion of normally non-phagocytic host cells. PLoS Pathog *2*, e129.

Franks, R.G., Wang, C., Levin, J.Z., and Liu, Z. (2002). SEUSS, a member of a novel family of plant regulatory proteins, represses floral homeotic gene expression with LEUNIG. Development *129*, 253-263.

Fuller, K.K., and Rhodes, J.C. (2012). Protein kinase A and fungal virulence: a sinister side to a conserved nutrient sensing pathway. Virulence *3*, 109-121.

Fuller, K.K., Richie, D.L., Feng, X., Krishnan, K., Stephens, T.J., Wikenheiser-Brokamp, K.A., Askew, D.S., and Rhodes, J.C. (2011). Divergent Protein Kinase A isoforms co-ordinately regulate conidial germination, carbohydrate metabolism and virulence in *Aspergillus fumigatus*. Mol Microbiol *79*, 1045-1062.

Gancedo, J.M. (2013). Biological roles of cAMP: variations on a theme in the different kingdoms of life. Biol Rev Camb Philos Soc *88*, 645-668.

Garzia, A., Etxebeste, O., Herrero-Garcia, E., Fischer, R., Espeso, E.A., and Ugalde, U. (2009). *Aspergillus nidulans* FlbE is an upstream developmental activator of conidiation functionally associated with the putative transcription factor FlbB. Mol Microbiol *71*, 172-184.

Garzia, A., Etxebeste, O., Herrero-Garcia, E., Ugalde, U., and Espeso, E.A. (2010). The concerted action of bZip and cMyb transcription factors FlbB and FlbD induces brlA expression and asexual development in *Aspergillus nidulans*. Mol Microbiol *75*, 1314-1324.

Gauthier, G.M., and Keller, N.P. (2013). Crossover fungal pathogens: the biology and pathogenesis of fungi capable of crossing kingdoms to infect plants and humans. Fungal Genet Biol *61*, 146-157.

Gavrias, V., Andrianopoulos, A., Gimeno, C.J., and Timberlake, W.E. (1996). *Saccharomyces cerevisiae TEC1* is required for pseudohyphal growth. Mol Microbiol *19*, 1255-1263.

Gehrke, A., Heinekamp, T., Jacobsen, I.D., and Brakhage, A.A. (2010). Heptahelical receptors GprC and GprD of *Aspergillus fumigatus* are essential regulators of colony growth, hyphal morphogenesis, and virulence. Appl Environ Microbiol *76*, 3989-3998.

Geiser, D.M., Klich, M.A., Frisvad, J.C., Peterson, S.W., Varga, J., and Samson, R.A. (2007). The current status of species recognition and identification in Aspergillus. Stud Mycol *59*, 1-10.

Gimeno, C.J., and Fink, G.R. (1994). Induction of pseudohyphal growth by overexpression of PHD1, a *Saccharomyces cerevisiae* gene related to transcriptional regulators of fungal development. Mol Cell Biol *14*, 2100-2112.

Gimeno, C.J., Ljungdahl, P.O., Styles, C.A., and Fink, G.R. (1992). Unipolar cell divisions in the yeast *S. cerevisiae* lead to filamentous growth: regulation by starvation and *RAS*. Cell *68*, 1077-1090.

Giraldo, M.C., and Valent, B. (2013). Filamentous plant pathogen effectors in action. Nat Rev Microbiol *11*, 800-814.

Gravelat, F.N., Beauvais, A., Liu, H., Lee, M.J., Snarr, B.D., Chen, D., Xu, W., Kravtsov, I., Hoareau, C.M., Vanier, G., Urb, M., Campoli, P., Al Abdallah, Q., Lehoux, M., Chabot, J.C., Ouimet, M.C., Baptista, S.D., Fritz, J.H., Nierman, W.C., Latgé, J.P., Mitchell, A.P., Filler, S.G., Fontaine, T., and Sheppard, D.C. (2013). *Aspergillus* galactosaminogalactan mediates adherence to host constituents and conceals hyphal β-glucan from the immune system. PLoS Pathog *9*, e1003575.

Gravelat, F.N., Ejzykowicz, D.E., Chiang, L.Y., Chabot, J.C., Urb, M., Macdonald, K.D., al-Bader, N., Filler, S.G., and Sheppard, D.C. (2010). *Aspergillus fumigatus*

MedA governs adherence, host cell interactions and virulence. Cell Microbiol *12*, 473-488.

Grice, C.M., Bertuzzi, M., and Bignell, E.M. (2013). Receptor-mediated signaling in *Aspergillus fumigatus*. Front Microbiol *4*, 26.

Grosse, C., Heinekamp, T., Kniemeyer, O., Gehrke, A., and Brakhage, A.A. (2008). Protein kinase A regulates growth, sporulation, and pigment formation in *Aspergillus fumigatus*. Appl Environ Microbiol *74*, 4923-4933.

Guarente, L., and Ptashne, M. (1981). Fusion of *Escherichia coli lacZ* to the cytochrome c gene of *Saccharomyces cerevisiae*. Proc Natl Acad Sci U S A *78*, 2199-2203.

Harcus, D., Nantel, A., Marcil, A., Rigby, T., and Whiteway, M. (2004). Transcription profiling of cyclic AMP signaling in *Candida albicans*. Mol Biol Cell *15*, 4490-4499.

Harting, R., Bayram, Ö., Laubinger, K., Valerius, O., and Braus, G.H. (2013). Interplay of the fungal sumoylation network for control of multicellular development. Mol Microbiol *90*, 1125-1145.

Hartmann, T., Cairns, T.C., Olbermann, P., Morschhauser, J., Bignell, E.M., and Krappmann, S. (2011). Oligopeptide transport and regulation of extracellular proteolysis are required for growth of *Aspergillus fumigatus* on complex substrates but not for virulence. Mol Microbiol *82*, 917-935.

Hartmann, T., Dümig, M., Jaber, B.M., Szewczyk, E., Olbermann, P., Morschhäuser, J., and Krappmann, S. (2010). Validation of a self-excising marker in the human pathogen *Aspergillus fumigatus* by employing the β-rec/six site-specific recombination system. Appl Environ Microbiol *76*, 6313-6317.

Hawksworth, D.L., and Rossman, A.Y. (1997). Where are all the undescribed fungi? Phytopathology *87*, 888-891.

Helmschrott, C., Sasse, A., Samantaray, S., Krappmann, S., and Wagener, J. (2013). Upgrading fungal gene expression on demand: improved systems for doxycycline-dependent silencing in *Aspergillus fumigatus*. Appl Environ Microbiol *79*, 1751-1754.

Herzog, B., Popova, B., Jakobshagen, A., Shahpasandzadeh, H., and Braus, G.H. (2013). Mutual cross talk between the regulators Hac1 of the unfolded protein response and Gcn4 of the general amino acid control of *Saccharomyces cerevisiae*. Eukaryot Cell *12*, 1142-1154.

Hogan, D.A., and Sundstrom, P. (2009). The Ras/cAMP/PKA signaling pathway and virulence in *Candida albicans*. Future Microbiol *4*, 1263-1270.

Hoyer, L.L., Green, C.B., Oh, S.H., and Zhao, X. (2008). Discovering the secrets of the *Candida albicans* agglutinin-like sequence (ALS) gene family—a sticky pursuit. Med Mycol *46*, 1-15.

Hube, B. (2009). Fungal adaptation to the host environment. Curr Opin Microbiol *12*, 347-349.

Hung, C.Y., Yu, J.J., Seshan, K.R., Reichard, U., and Cole, G.T. (2002). A parasitic phase-specific adhesin of *Coccidioides immitis* contributes to the virulence of this respiratory Fungal pathogen. Infect Immun *70*, 3443-3456.

Hunter, S., Jones, P., Mitchell, A., Apweiler, R., Attwood, T.K., Bateman, A., Bernard, T., Binns, D., Bork, P., Burge, S., de Castro, E., Coggill, P., Corbett, M., Das, U., Daugherty, L., Duquenne, L., Finn, R.D., Fraser, M., Gough, J., Haft, D., Hulo, N., Kahn, D., Kelly, E., Letunic, I., Lonsdale, D., Lopez, R., Madera, M., Maslen, J., McAnulla, C., McDowall, J., McMenamin, C., Mi, H., Mutowo-Muellenet, P., Mulder, N., Natale, D., Orengo, C., Pesseat, S., Punta, M., Quinn, A.F., Rivoire, C., Sangrador-Vegas, A., Selengut, J.D., Sigrist, C.J., Scheremetjew, M., Tate, J., Thimmajanarthanan, M., Thomas, P.D., Wu, C.H., Yeats, C., and Yong, S.Y. (2012). InterPro in 2011: new developments in the family and domain prediction database. Nucleic Acids Res *40*, D306-312.

Inglis, D.O., and Sherlock, G. (2013). Ras signaling gets fine-tuned: regulation of multiple pathogenic traits of *Candida albicans*. Eukaryot Cell *12*, 1316-1325.

Inoue, H., Nojima, H., and Okayama, H. (1990). High efficiency transformation of *Escherichia coli* with plasmids. Gene *96*, 23-28.

Ito, H., Fukuda, Y., Murata, K., and Kimura, A. (1983). Transformation of intact yeast cells treated with alkali cations. J Bacteriol *153*, 163-168.

Jacobsen, I.D., Große, K., and Hube, B. (2012). Embryonated chicken eggs as alternative infection model for pathogenic fungi. Methods Mol Biol *845*, 487-496.

Jacobsen, I.D., Große, K., Slesiona, S., Hube, B., Berndt, A., and Brock, M. (2010). Embryonated eggs as an alternative infection model to investigate *Aspergillus fumigatus* virulence. Infect Immun *78*, 2995-3006.

Jensen, B.G., Andersen, M.R., Pedersen, M.H., Frisvad, J.C., and Sondergaard, I. (2010). Hydrophobins from *Aspergillus* species cannot be clearly divided into two classes. BMC Res Notes *3*, 344.

Johnston, M., and Davis, R.W. (1984). Sequences that regulate the divergent *GAL1-GAL10* promoter in *Saccharomyces cerevisiae*. Mol Cell Biol *4*, 1440-1448.

Käfer, E. (1977). Meiotic and mitotic recombination in Aspergillus and its chromosomal aberrations. Adv Genet *19*, 33-131.

Kim, S., Ahn, I.P., Rho, H.S., and Lee, Y.H. (2005). MHP1, a *Magnaporthe grisea* hydrophobin gene, is required for fungal development and plant colonization. Mol Microbiol *57*, 1224-1237.

Kim, T.S., Kim, H.Y., Yoon, J.H., and Kang, H.S. (2004). Recruitment of the Swi/Snf complex by Ste12-Tec1 promotes Flo8-Mss11-mediated activation of STA1 expression. Mol Cell Biol *24*, 9542-9556.

Klimes, A., and Dobinson, K.F. (2006). A hydrophobin gene, VDH1, is involved in microsclerotial development and spore viability in the plant pathogen *Verticillium dahliae*. Fungal Genet Biol *43*, 283-294.

Kobayashi, O., Suda, H., Ohtani, T., and Sone, H. (1996). Molecular cloning and analysis of the dominant flocculation gene *FLO8* from *Saccharomyces cerevisiae*. Mol Gen Genet *251*, 707-715.

Koch, B.J., Ryan, J.F., and Baxevanis, A.D. (2012). The diversification of the LIM superclass at the base of the metazoa increased subcellular complexity and promoted multicellular specialization. PLoS One *7*, e33261.

Kong, Q., Wang, L., Liu, Z., Kwon, N.J., Kim, S.C., and Yu, J.H. (2013). Gbeta-like CpcB plays a crucial role for growth and development of *Aspergillus nidulans* and *Aspergillus fumigatus*. PLoS One *8*, e70355.

Kozubowski, L., Lee, S.C., and Heitman, J. (2009). Signalling pathways in the pathogenesis of *Cryptococcus*. Cell Microbiol *11*, 370-380.

Krappmann, S., and Braus, G.H. (2005). Nitrogen metabolism of Aspergillus and its role in pathogenicity. Med Mycol *43 Suppl 1*, S31-40.

Krappmann, S., Sasse, C., and Braus, G.H. (2006). Gene targeting in *Aspergillus fumigatus* by homologous recombination is facilitated in a nonhomologous end-joining-deficient genetic background. Eukaryot Cell *5*, 212-215.

Krijgsheld, P., Bleichrodt, R., van Veluw, G.J., Wang, F., Müller, W.H., Dijksterhuis, J., and Wösten, H.A. (2013). Development in Aspergillus. Stud Mycol *74*, 1-29.

Kubodera, T., Yamashita, N., and Nishimura, A. (2000). Pyrithiamine resistance gene (*ptrA*) of *Aspergillus oryzae*: cloning, characterization and application as a dominant selectable marker for transformation. Biosci Biotechnol Biochem *64*, 1416-1421.

Kweon, Y., Rothe, A., Conibear, E., and Stevens, T.H. (2003). Ykt6p is a multifunctional yeast R-SNARE that is required for multiple membrane transport pathways to the vacuole. Mol Biol Cell *14*, 1868-1881.

Kwon, N.J., Garzia, A., Espeso, E.A., Ugalde, U., and Yu, J.H. (2010a). FlbC is a putative nuclear C2H2 transcription factor regulating development in *Aspergillus nidulans*. Mol Microbiol *77*, 1203-1219.

Kwon, N.J., Shin, K.S., and Yu, J.H. (2010b). Characterization of the developmental regulator FlbE in *Aspergillus fumigatus* and *Aspergillus nidulans*. Fungal Genet Biol *47*, 981-993.

Lafon, A., Han, K.H., Seo, J.A., Yu, J.H., and d'Enfert, C. (2006). G-protein and cAMP-mediated signaling in aspergilli: a genomic perspective. Fungal Genet Biol *43*, 490-502.

Lamarre, C., Beau, R., Balloy, V., Fontaine, T., Wong Sak Hoi, J., Guadagnini, S., Berkova, N., Chignard, M., Beauvais, A., and Latgé, J.P. (2009). Galactofuranose attenuates cellular adhesion of *Aspergillus fumigatus*. Cell Microbiol *11*, 1612-1623.

Lee, B., and Taylor, J. (1990). Isolation of DNA from fungal mycelia and single spores. San Diego: Academic Press Inc.

Lee, B.Y., Han, S.Y., Choi, H.G., Kim, J.H., Han, K.H., and Han, D.M. (2005). Screening of growth- or development-related genes by using genomic library with inducible promoter in *Aspergillus nidulans*. J Microbiol *43*, 523-528.

Lee, M.K., Kwon, N.J., Choi, J.M., Lee, I.S., Jung, S., and Yu, J.H. (2014). NsdD Is a Key Repressor of Asexual Development in *Aspergillus nidulans*. Genetics. DOI 10.1534/genetics.114.161430

Lengeler, K.B., Davidson, R.C., D'Souza, C., Harashima, T., Shen, W.C., Wang, P., Pan, X., Waugh, M., and Heitman, J. (2000). Signal transduction cascades regulating fungal development and virulence. Microbiol Mol Biol Rev *64*, 746-785.

Levdansky, E., Kashi, O., Sharon, H., Shadkchan, Y., and Osherov, N. (2010). The *Aspergillus fumigatus cspA* gene encoding a repeat-rich cell wall protein is important for normal conidial cell wall architecture and interaction with host cells. Eukaryot Cell *9*, 1403-1415.

Levdansky, E., Romano, J., Shadkchan, Y., Sharon, H., Verstrepen, K.J., Fink, G.R., and Osherov, N. (2007). Coding tandem repeats generate diversity in *Aspergillus fumigatus* genes. Eukaryot Cell *6*, 1380-1391.

Li, L., Wright, S.J., Krystofova, S., Park, G., and Borkovich, K.A. (2007). Heterotrimeric G protein signaling in filamentous fungi. Annu Rev Microbiol *61*, 423-452.

Liebmann, B., Gattung, S., Jahn, B., and Brakhage, A.A. (2003). cAMP signaling in *Aspergillus fumigatus* is involved in the regulation of the virulence gene *pksP*

and in defense against killing by macrophages. Mol Genet Genomics *269*, 420-435.

Liebmann, B., Müller, M., Braun, A., and Brakhage, A.A. (2004). The cyclic AMP-dependent protein kinase a network regulates development and virulence in *Aspergillus fumigatus*. Infect Immun *72*, 5193-5203.

Lilly, L.M., Scopel, M., Nelson, M.P., Burg, A.R., Dunaway, C.W., and Steele, C. (2014). Eosinophil Deficiency Compromises Lung Defense against *Aspergillus fumigatus*. Infect Immun *82*, 1315-1325.

Linder, M.B., Szilvay, G.R., Nakari-Setala, T., and Penttila, M.E. (2005). Hydrophobins: the protein-amphiphiles of filamentous fungi. FEMS Microbiol Rev *29*, 877-896.

Linder, T., and Gustafsson, C.M. (2008). Molecular phylogenetics of ascomycotal adhesins--a novel family of putative cell-surface adhesive proteins in fission yeasts. Fungal Genet Biol *45*, 485-497.

Liu, H., Styles, C.A., and Fink, G.R. (1996). *Saccharomyces cerevisiae* S288C has a mutation in *FLO8*, a gene required for filamentous growth. Genetics *144*, 967-978.

Livak, K.J., and Schmittgen, T.D. (2001). Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. Methods *25*, 402-408.

Loussert, C., Schmitt, C., Prevost, M.C., Balloy, V., Fadel, E., Philippe, B., Kauffmann-Lacroix, C., Latgé, J.P., and Beauvais, A. (2010). *In vivo* biofilm composition of *Aspergillus fumigatus*. Cell Microbiol *12*, 405-410.

Müller, P., Leibbrandt, A., Teunissen, H., Cubasch, S., Aichinger, C., and Kahmann, R. (2004). The Gβ-subunit-encoding gene *bpp1* controls cyclic-AMP signaling in *Ustilago maydis*. Eukaryot Cell *3*, 806-814.

Mah, J.H., and Yu, J.H. (2006). Upstream and downstream regulation of asexual development in *Aspergillus fumigatus*. Eukaryot Cell *5*, 1585-1595.

Marshall, M.A., and Timberlake, W.E. (1991). *Aspergillus nidulans wetA* activates spore-specific gene expression. Mol Cell Biol *11*, 55-62.

Mayer, F.L., Wilson, D., and Hube, B. (2013). *Candida albicans* pathogenicity mechanisms. Virulence *4*, 119-128.

McDonough, K.A., and Rodriguez, A. (2012). The myriad roles of cyclic AMP in microbial pathogens: from signal to sword. Nat Rev Microbiol *10*, 27-38.

Mitchell, T.K., and Dean, R.A. (1995). The cAMP-dependent protein kinase catalytic subunit is required for appressorium formation and pathogenesis by the rice blast pathogen *Magnaporthe grisea*. Plant Cell *7*, 1869-1878.

Morris, G., Kokki, M.H., Anderson, K., and Richardson, M.D. (2000). Sampling of Aspergillus spores in air. J Hosp Infect *44*, 81-92.

Mumberg, D., Müller, R., and Funk, M. (1994). Regulatable promoters of *Saccharomyces cerevisiae*: comparison of transcriptional activity and their use for heterologous expression. Nucleic Acids Res *22*, 5767-5768.

Neer, E.J., Schmidt, C.J., Nambudripad, R., and Smith, T.F. (1994). The ancient regulatory-protein family of WD-repeat proteins. Nature *371*, 297-300.

Neves, S.R., Ram, P.T., and Iyengar, R. (2002). G protein pathways. Science *296*, 1636-1639.

Ni, M., and Yu, J.H. (2007). A novel regulator couples sporogenesis and trehalose biogenesis in *Aspergillus nidulans*. PLoS One *2*, e970.

Octavio, L.M., Gedeon, K., and Maheshri, N. (2009). Epigenetic and conventional regulation is distributed among activators of *FLO11* allowing tuning of population-level heterogeneity in its expression. PLoS Genet *5*, e1000673.

Oliver, B.G., Panepinto, J.C., Askew, D.S., and Rhodes, J.C. (2002). cAMP alteration of growth rate of *Aspergillus fumigatus* and *Aspergillus niger* is carbon-source dependent. Microbiology *148*, 2627-2633.

Ortoneda, M., Guarro, J., Madrid, M.P., Caracuel, Z., Roncero, M.I., Mayayo, E., and Di Pietro, A. (2004). *Fusarium oxysporum* as a multihost model for the genetic dissection of fungal virulence in plants and mammals. Infect Immun *72*, 1760-1766.

Pan, X., and Heitman, J. (1999). Cyclic AMP-dependent protein kinase regulates pseudohyphal differentiation in *Saccharomyces cerevisiae*. Mol Cell Biol *19*, 4874-4887.

Pan, X., and Heitman, J. (2002). Protein kinase A operates a molecular switch that governs yeast pseudohyphal differentiation. Mol Cell Biol *22*, 3981-3993.

Paris, S., Debeaupuis, J.P., Crameri, R., Carey, M., Charles, F., Prevost, M.C., Schmitt, C., Philippe, B., and Latgé, J.P. (2003). Conidial hydrophobins of *Aspergillus fumigatus*. Appl Environ Microbiol *69*, 1581-1588.

Park, H.S., Bayram, Ö., Braus, G.H., Kim, S.C., and Yu, J.H. (2012). Characterization of the velvet regulators in *Aspergillus fumigatus*. Mol Microbiol *86*, 937-953.

Park, H.S., Nam, T.Y., Han, K.H., Kim, S.C., and Yu, J.H. (2014). VelC Positively Controls Sexual Development in *Aspergillus nidulans*. PLoS One *9*, e89883.

Park, H.S., and Yu, J.H. (2012). Genetic control of asexual sporulation in filamentous fungi. Curr Opin Microbiol *15*, 669-677.

Parta, M., Chang, Y., Rulong, S., Pinto-DaSilva, P., and Kwon-Chung, K.J. (1994). *HYP1*, a hydrophobin gene from *Aspergillus fumigatus*, complements the *rodletless* phenotype in *Aspergillus nidulans*. Infect Immun *62*, 4389-4395.

Prade, R.A., and Timberlake, W.E. (1993). The *Aspergillus nidulans brlA* regulatory locus consists of overlapping transcription units that are individually required for conidiophore development. EMBO J *12*, 2439-2447.

Ramana, J., and Gupta, D. (2010). FaaPred: a SVM-based prediction method for fungal adhesins and adhesin-like proteins. PLoS One *5*, e9695.

Ramanujam, R., and Naqvi, N.I. (2010). PdeH, a high-affinity cAMP phosphodiesterase, is a key regulator of asexual and pathogenic differentiation in *Magnaporthe oryzae*. PLoS Pathog *6*, e1000897.

Rigden, D.J., Mello, L.V., and Galperin, M.Y. (2004). The PA14 domain, a conserved all-beta domain in bacterial toxins, enzymes, adhesins and signaling molecules. Trends Biochem Sci *29*, 335-339.

Robert, V.A., and Casadevall, A. (2009). Vertebrate endothermy restricts most fungi as potential pathogens. J Infect Dis *200*, 1623-1626.

Rohde, M., and Chhatwal, G.S. (2013). Adherence and invasion of streptococci to eukaryotic cells and their role in disease pathogenesis. Curr Top Microbiol Immunol *368*, 83-110.

Rose, M., and Botstein, D. (1983). Construction and use of gene fusions to *lacZ* (β-galactosidase) that are expressed in yeast. Methods Enzymol *101*, 167-180.

Rupp, S., Summers, E., Lo, H.J., Madhani, H., and Fink, G. (1999). MAP kinase and cAMP filamentation signaling pathways converge on the unusually large promoter of the yeast *FLO11* gene. EMBO J *18*, 1257-1269.

Ryan, O., Shapiro, R.S., Kurat, C.F., Mayhew, D., Baryshnikova, A., Chin, B., Lin, Z.Y., Cox, M.J., Vizeacoumar, F., Cheung, D., Bahr, S., Tsui, K., Tebbji, F., Sellam, A., Istel, F., Schwarzmüller, T., Reynolds, T.B., Kuchler, K., Gifford, D.K., Whiteway, M., Giaever, G., Nislow, C., Costanzo, M., Gingras, A.C., Mitra, R.D., Andrews, B., Fink, G.R., Cowen, L.E., and Boone, C. (2012). Global gene deletion analysis exploring yeast filamentous growth. Science *337*, 1353-1356.

Sambrook, J., Fritsch, E., and Maniatis, T. (1989). Molecular Cloning: A Laboratory Manual. New York: Cold Spring Harbor Laboratory Press.

Sangsoda, S., Cherest, H., and Surdin-Kerjan, Y. (1985). The expression of the *MET25* gene of *Saccharomyces cerevisiae* is regulated transcriptionally. Mol Gen Genet *200*, 407-414.

Schobel, F., Jacobsen, I.D., and Brock, M. (2010). Evaluation of lysine biosynthesis as an antifungal drug target: biochemical characterization of *Aspergillus fumigatus* homocitrate synthase and virulence studies. Eukaryot Cell *9*, 878-893.

Sevim, A., Donzelli, B.G., Wu, D., Demirbag, Z., Gibson, D.M., and Turgeon, B.G. (2012). Hydrophobin genes of the entomopathogenic fungus, *Metarhizium brunneum*, are differentially expressed and corresponding mutants are decreased in virulence. Curr Genet *58*, 79-92.

Sewall, T.C., Mims, C.W., and Timberlake, W.E. (1990). *abaA* controls phialide differentiation in *Aspergillus nidulans*. Plant Cell *2*, 731-739.

Sexton, A.C., and Howlett, B.J. (2006). Parallels in fungal pathogenesis on plant and animal hosts. Eukaryot Cell *5*, 1941-1949.

Shapiro, R.S., Ryan, O., Boone, C., and Cowen, L.E. (2012). Regulatory circuitry governing morphogenesis in *Saccharomyces cerevisiae* and *Candida albicans*. Cell Cycle *11*, 4294-4295.

Sheppard, D.C. (2011). Molecular mechanism of *Aspergillus fumigatus* adherence to host constituents. Curr Opin Microbiol *14*, 375-379.

Sheppard, D.C., Doedt, T., Chiang, L.Y., Kim, H.S., Chen, D., Nierman, W.C., and Filler, S.G. (2005). The *Aspergillus fumigatus* StuA protein governs the up-regulation of a discrete transcriptional program during the acquisition of developmental competence. Mol Biol Cell *16*, 5866-5879.

Shevchenko, A., Wilm, M., Vorm, O., and Mann, M. (1996). Mass spectrometric sequencing of proteins silver-stained polyacrylamide gels. Anal Chem *68*, 850-858.

Siemens, N., Patenge, N., Otto, J., Fiedler, T., and Kreikemeyer, B. (2011). *Streptococcus pyogenes* M49 plasminogen/plasmin binding facilitates keratinocyte invasion via integrin-integrin-linked kinase (ILK) pathways and protects from macrophage killing. J Biol Chem *286*, 21612-21622.

Southern, E.M. (1975). Detection of specific sequences among DNA fragments separated by gel electrophoresis. J Mol Biol *98*, 503-517.

Southern, P., Horbul, J., Maher, D., and Davis, D.A. (2008). *C. albicans* colonization of human mucosal surfaces. PLoS One *3*, e2067.

Stoldt, V., Rademacher, F., Kehren, V., Ernst, J.F., Pearce, D.A., and Sherman, F. (1996). Review: the Cct eukaryotic chaperonin subunits of *Saccharomyces cerevisiae* and other yeasts. Yeast *12*, 523-529.

Su, C., Li, Y., Lu, Y., and Chen, J. (2009). Mss11, a transcriptional activator, is required for hyphal development in *Candida albicans*. Eukaryot Cell *8*, 1780-1791.

Talbot, N.J., Ebbole, D.J., and Hamer, J.E. (1993). Identification and characterization of *MPG1*, a gene involved in pathogenicity from the rice blast fungus *Magnaporthe grisea*. Plant Cell *5*, 1575-1590.

Talbot, N.J., Kershaw, M.J., Wakley, G.E., De Vries, O., Wessels, J., and Hamer, J.E. (1996). *MPG1* Encodes a Fungal Hydrophobin Involved in Surface Interactions during Infection-Related Development of *Magnaporthe grisea*. Plant Cell *8*, 985-999.

Tao, L., and Yu, J.H. (2011). AbaA and WetA govern distinct stages of *Aspergillus fumigatus* development. Microbiology *157*, 313-326.

Tekaia, F., and Latgé, J.P. (2005). *Aspergillus fumigatus*: saprophyte or pathogen? Curr Opin Microbiol *8*, 385-392.

Thau, N., Monod, M., Crestani, B., Rolland, C., Tronchin, G., Latgé, J.P., and Paris, S. (1994). rodletless mutants of *Aspergillus fumigatus*. Infect Immun *62*, 4380-4388.

Tollervey, D., Lehtonen, H., Carmo-Fonseca, M., and Hurt, E.C. (1991). The small nucleolar RNP protein NOP1 (fibrillarin) is required for pre-rRNA processing in yeast. EMBO J *10*, 573-583.

Tran, V.T., Braus-Stromeyer, S.A., Kusch, H., Reusche, M., Kaever, A., Kühn, A., Valerius, O., Landesfeind, M., Aßhauer, K., Tech, M., Hoff, K., Pena-Centeno, T., Stanke, M., Lipka, V., and Braus, G.H. (2014). *Verticillium* transcription activator of adhesion Vta2 suppresses microsclerotia formation and is required for systemic infection of plant roots. New Phytol *202*, 565-581.

Twumasi-Boateng, K., Yu, Y., Chen, D., Gravelat, F.N., Nierman, W.C., and Sheppard, D.C. (2009). Transcriptional profiling identifies a role for BrlA in the response to nitrogen depletion and for StuA in the regulation of secondary metabolite clusters in *Aspergillus fumigatus*. Eukaryot Cell *8*, 104-115.

Upadhyay, S.K., Mahajan, L., Ramjee, S., Singh, Y., Basir, S.F., and Madan, T. (2009). Identification and characterization of a laminin-binding protein of *Aspergillus fumigatus*: extracellular thaumatin domain protein (AfCalAp). J Med Microbiol *58*, 714-722.

Uppuluri, P., and Lopez-Ribot, J.L. (2010). An easy and economical in vitro method for the formation of *Candida albicans* biofilms under continuous conditions of flow. Virulence *1*, 483-487.

van Meyel, D.J., Thomas, J.B., and Agulnick, A.D. (2003). Ssdp proteins bind to LIM-interacting co-factors and regulate the activity of LIM-homeodomain protein complexes in vivo. Development *130*, 1915-1925.

Verstrepen, K.J., and Klis, F.M. (2006). Flocculation, adhesion and biofilm formation in yeasts. Mol Microbiol *60*, 5-15.

Verstrepen, K.J., Reynolds, T.B., and Fink, G.R. (2004). Origins of variation in the fungal cell surface. Nat Rev Microbiol *2*, 533-540.

Vinh, D.B., and Drubin, D.G. (1994). A yeast TCP-1-like protein is required for actin function in vivo. Proc Natl Acad Sci U S A *91*, 9116-9120.

von Zeska Kress, M.R., Harting, R., Bayram, Ö., Christmann, M., Irmer, H., Valerius, O., Schinke, J., Goldman, G.H., and Braus, G.H. (2012). The COP9 signalosome counteracts the accumulation of cullin SCF ubiquitin E3 RING ligases during fungal development. Mol Microbiol *83*, 1162-1177.

Wang, C., and St Leger, R.J. (2007). The MAD1 adhesin of *Metarhizium anisopliae* links adhesion with blastospore production and virulence to insects, and the MAD2 adhesin enables attachment to plants. Eukaryot Cell *6*, 808-816.

Wang, L., Zhai, B., and Lin, X. (2012). The link between morphotype transition and virulence in *Cryptococcus neoformans*. PLoS Pathog *8*, e1002765.

Whiteway, M., and Oberholzer, U. (2004). *Candida* morphogenesis and host-pathogen interactions. Curr Opin Microbiol *7*, 350-357.

Wieser, J., Lee, B.N., Fondon, J., 3rd, and Adams, T.H. (1994). Genetic requirements for initiating asexual development in *Aspergillus nidulans*. Curr Genet *27*, 62-69.

Woodcock, D.M., Crowther, P.J., Doherty, J., Jefferson, S., DeCruz, E., Noyer-Weidner, M., Smith, S.S., Michael, M.Z., and Graham, M.W. (1989). Quantitative evaluation of *Escherichia coli* host strains for tolerance to cytosine methylation in plasmid and phage recombinants. Nucleic Acids Res *17*, 3469-3478.

Woolhouse, M., and Gaunt, E. (2007). Ecological origins of novel human pathogens. Crit Rev Microbiol *33*, 231-242.

Wu, J., and Miller, B.L. (1997). *Aspergillus* asexual reproduction and sexual reproduction are differentially affected by transcriptional and translational mechanisms regulating stunted gene expression. Mol Cell Biol *17*, 6191-6201.

Xiao, P., Shin, K.S., Wang, T., and Yu, J.H. (2010). *Aspergillus fumigatus flbB* encodes two basic leucine zipper domain (bZIP) proteins required for proper asexual development and gliotoxin production. Eukaryot Cell *9*, 1711-1723.

Yan, X., Li, Y., Yue, X., Wang, C., Que, Y., Kong, D., Ma, Z., Talbot, N.J., and Wang, Z. (2011). Two novel transcriptional regulators are essential for infection-related morphogenesis and pathogenicity of the rice blast fungus *Magnaporthe oryzae*. PLoS Pathog *7*, e1002385.

Yelton, M.M., Hamer, J.E., and Timberlake, W.E. (1984). Transformation of *Aspergillus nidulans* by using a *trpC* plasmid. Proc Natl Acad Sci U S A *81*, 1470-1474.

Yu, J.H. (2010). Regulation of Development in *Aspergillus nidulans* and *Aspergillus fumigatus*. Mycobiology *38*, 229-237.

Yu, J.H., Wieser, J., and Adams, T.H. (1996). The *Aspergillus* FlbA RGS domain protein antagonizes G protein signaling to block proliferation and allow development. EMBO J *15*, 5184-5190.

Zhang, S., Xia, Y.X., Kim, B., and Keyhani, N.O. (2011). Two hydrophobins are involved in fungal spore coat rodlet layer assembly and each play distinct roles in surface interactions, development and pathogenesis in the entomopathogenic fungus, *Beauveria bassiana*. Mol Microbiol *80*, 811-826.

Zhao, W., Panepinto, J.C., Fortwendel, J.R., Fox, L., Oliver, B.G., Askew, D.S., and Rhodes, J.C. (2006). Deletion of the regulatory subunit of protein kinase A in *Aspergillus fumigatus* alters morphology, sensitivity to oxidative damage, and virulence. Infect Immun *74*, 4865-4874.

Abbreviations

°C	degree centigrade
APS	ammonium persulfate
ATP	adenosine triphosphate
APSES	Asm1, Phd1, Sok2, Efg1 and StuA
BLAST	basic local alignment search tool
bp	base pair
CAM	chorioallantoic membrane
cAMP	cyclic adenosine monophosphate
cDNA	complementary DNA
C-terminus	carboxyl terminus
Δ	deletion
D	Germany
DNA	deoxyribonucleic acid
DTT	dithiothreitol
EDTA	ethylenediaminetetraacetic acid
Flo	flocculins
GPCR	G protein coupled receptor
GPI	glycosyl phosphatidyl inositol
h	hour(s)
kb	kilobase(s)
kDa	kilo Dalton
l	liter
LB	lysogenic broth
LCMS	liquid chromatography mass spectrometry

Leu	L-leucine
LisH	Lis homology
LUFS	LUG/LUH-Flo8-single-stranded
M	molar
Met	L-methionine
mg	milli-gram
min	minute(s)
ml	milli-liter
μl	micro-liter
mm	milli-meter
μm	micro-meter
mM	milli-molar
MM	minimal medium
mRNA	messenger ribonucleic acid
N-terminus	NH2 terminus
NLS	nuclear localization signal
OD	optical density
PBS	phosphate buffer saline
PCR	polymerase chain reaction
PKA	protein kinase A
ptrA	pyridthyamin
qRT	quantitative real-time
RNA	ribonucleic acid
RT	room temperature
rpm	revolutions per minute

s	second(s)
SC	synthetic complete
sGFP	synthetic green fluorescent protein
SLAD	synthetic low ammonium dextrose
UDP	uridine diphosphate
Ura	uracil

List of figures

Acknowledgement

I would like to thank Prof. Dr. Gerhard H. Braus for supervision and giving me the opportunity to do my PhD thesis in his department. I appreciate his advice and time for my project and thesis.

I want to thanks Prof. Dr. Stefanie Pöggeler, Prof. Dr. Uwe Groß and Prof. Dr. Utz Reichard for being in my thesis committee and their suggestions in all the meetings.

I would like to thank Dr. Henriette Irmer for her support during my PhD thesis and for her guidance and helpful discussions.

I am particularly thankful for Dr. Christoph Sasse and Bastian Jöhnk for their experiences, ideas and their time for discussions and proof reading of my thesis. I would like to thank Dr. Blaga Popova, Alexandra Juckert, Hedieh Shapasandzadeh for the great atmosphere in the lab and proof reading of my thesis. I am thankful for Drs. Van Tuan Tran and Britta Herzog and Maria Meyer for their contribution to the yeast experiments, Dr. Jennifer Gerke for HLPC analysis, Dr. Oliver Valerius for LCMS analysis and MaxQuant software. I would like to thank Sabine Reen for reading my thesis.

I also want to thank Heidi Northemann and Nicole Scheiter for official documents and chemical supply, Andrea Wäge for preparing experimental wares. I am thankful for Dr. Mirit Kolog-Gulko for personal support and scientific ideas. Furthermore, I am particularly thankful for the helps from all people of this department.

Lastly, I would like to thank my family and friends for their endless support and help. Especially, I am thankful for friends living in Göttingen for sharing the life experiences and their general support.

Curriculum vitae

Personal details

Chi-Jan Lin

Born on May 7th, 1985

In Hsinchu, Taiwan

Education

1991 – 2000	Elementary and junior education in Chutung, Hsinchu, Taiwan
2000 – 2003	High school education at Hsinchu High school in Hsinchu, Taiwan

Scientific background

2003 – 2007	Bachelor of Science in Life Science at the National Tsing-Hua University, Hsinchu, Taiwan
2007 – 2009	Master of Science in Life Science at the National Tsing-Hua University, Hsinchu, Taiwan Master thesis „Widespread dissemination of aminoglycoside and quinolone resistance in CTX-M type extended spectrum β-lactamases producing *Klebsiella pneumoniae* in Taiwan" in the Division of Infectious Diseases at National Health Research Institutes, Miaoli, Taiwan
2011 – 2014	Scientific assistant and PhD student in the lab of Prof. Gerhard Braus at the Georg-August University Göttingen