The Transcriptional Regulator HbxA Governs Development, Secondary Metabolism, and Virulence in Aspergillus fumigatus

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The transcriptional regulator HbxA governs development, secondary metabolism and virulence in *Aspergillus fumigatus*

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Abstract

Aspergillus fumigatus is the leading cause of Invasive Aspergillosis which in immunocompromised patients results in a mortality rate as high as 90%. Earlier studies showed that HbxA is a global regulator in *A. flavus* affecting morphological development and secondary metabolism. Here we determined its role in *A. fumigatus* examining whether HbxA influences regulation of asexual development, natural product biosynthesis, and virulence of this fungus. Our analysis demonstrated that removal of the *hbxA* gene caused a near complete loss of conidial production in the mutant strain, as well as a slight reduction in colony growth. Other aspects of asexual development are affected, such as size and germination of conidia. Furthermore, we showed that in *A. fumigatus* loss of *hbxA* decreased the expression of the *brlA* central regulatory

pathway involved in asexual development, as well as the expression of the "fluffy" genes *flbB*, *flbD*, and *fluG*. HbxA was also found to regulate secondary metabolism, affecting the biosynthesis of multiple natural products, including fumigaclavines, fumiquinazolines, and chaetomine. In addition, using a neutropenic mouse infection model *hbxA* was found to negatively impact the virulence of *A. fumigatus*.

Importance of work

The number of immunodepressed individuals is increasing, mainly due to the greater life expectancy in immunodepressed patients due to improvements in modern medical treatments. However, this population group is highly susceptible to Invasive Aspergillosis. This devastating illness, mainly caused by the fungus *Aspergillus fumigatus*, is associated with mortality rates reaching 90%. Treatment options for this disease are currently limited, and a better understanding of *A. fumigatus* genetic regulatory mechanisms is paramount for the design of new strategies to prevent or combat this infection. Our work provides new insight into the regulation of the development, metabolism and virulence of this important opportunistic pathogen. The transcriptional regulatory gene *hbxA* has a profound effect on *A. fumigatus* biology, governing multiple aspects of conidial development. This is relevant since conidia are the main source of inoculum in *Aspergillus* infections. Importantly, *hbxA* also regulates the biosynthesis of secondary metabolites and pathogenicity of this fungus.

Introduction

The opportunistic human pathogen *Aspergillus fumigatus* is a known cause of a wide range of illnesses, including Invasive Aspergillosis, (IA). Immunodeficient individuals are

48 particularly susceptive to these diseases [1, 2]. This population group includes organ transplant
49 patients, individuals with genetic immunodeficiencies or receiving chemotherapy, and HIV
50 patients [3-8]. The main site of entry leading to *A. fumigatus* infections is the respiratory tract.
51 Healthy individuals are able to eliminate the inhaled fungal conidia (asexual spores) through
52 mucociliary clearance. The remaining fungal spores encounter epithelial cells or alveolar
53 macrophages, responsible for phagocytosis and killing of spores, and for the initiation of a
54 proinflammatory response that recruits neutrophils. Neutrophils are able to destroy hyphae from
55 germinated conidia that evaded macrophages. However, patients who are neutropenic are at high
56 risk of developing IA, with a mortality rate of up to 90 % [9-13].
57 The small size of the *A. fumigatus* conidia contributes to its pathogenicity, reaching the
58 lung alveoli and establishing an infection that can become systemic [2]. In addition, *A. fumigatus*
59 produces numerous secondary metabolites [14-21], that are considered part of the fungal
60 chemical arsenal required for niche specialization [22] including host-fungus interactions. Some
61 of these metabolites act as immunosuppressants, which may be in association with pathogenic
62 processes. For most IA infections early diagnosis is critical, followed by treatment with
63 antifungal drugs such as azoles, typically voriconazole [23-24]. Recently, strains of *A. fumigatus*
64 have been shown to gain drug resistance, thus it is necessary to identify new targets to control or
65 prevent the potentially lethal infections caused by *A. fumigatus* [24].
66 Fungal regulatory genes could constitute some of these novel genetic targets to design
67 antifungal therapies. In this work we focused on studying the homeobox transcriptional regulator
68 gene *hbxA*. Homeobox (Hbx) proteins are a class of transcriptional regulators that govern
69 development in many eukaryotes including animals, plants and other fungi [25]. A homolog of
70 this gene in *Aspergillus flavus* has been shown to regulate aspects of morphological

71 differentiation, including asexual development [26]. A recent *hbx1*-dependent transcriptome
72 analysis revealed that this gene controls numerous genes involved in morphogenesis and
73 secondary metabolism [27]. We hypothesize that the homolog of *hbx1* in *A. fumigatus, hbxA,*
74 may play a similar role in the regulation of development and secondary metabolism in this
75 important opportunistic human pathogen.

76 Our study revealed that in *A. fumigatus* lack of *hbxA* leads to a slight reduction in colony
77 growth and a near complete loss of conidial production. Furthermore, we showed that loss of
78 *hbxA* decreased the expression of genes in the *brlA*-central developmental pathway, as well as
79 the expression of the "fluffy" genes *flbB*, *flbD*, and *fluG*. Other aspects of asexual development
80 were affected in the absence of *hbxA*, such as the size and germination rate of conidia. In
81 addition, metabolomics analysis of the *hbxA* mutant indicated that this gene is a master regulator
82 that governs the biosynthesis of numerous natural products. With respect to the effect of *hbxA* on
83 pathogenicity, infection of neutropenic mice showed an increase in virulence in the *hbxA*
84 deletion mutant compared to the *A. fumigatus* wild-type strain.
85

86 ## Results

87 ***hbxA* is required for normal fungal growth and asexual development in *A. fumigatus.***

88 To determine the role of *hbxA* in *A. fumigatus,* two strains were generated, an *hbxA*
89 deletion and complementation strain, (Figure S1A and S1B). Deletion of *hbxA* was confirmed by
90 diagnostic PCR, yielding the expected 2.8 kb PCR product. The complementation strain was also
91 verified by PCR, producing a 2.7 kb DNA fragment. In addition, the strains were confirmed by
92 assessing *hbxA* expression levels with qRT-PCR (Figure S1C). *hbxA* transcripts were absent in
93 the Δ*hbxA* strain, while the complementation strain showed a restoration of *hbxA* expression

similar to that of the wild type. Next, the growth of all the strains was evaluated in point-inoculated cultures growing on solid GMM for seven days. Our results indicated an approximately 1.4-fold reduction in the growth of Δ*hbxA* compared to the controls (Figure 1).

Absence of *hbxA* resulted in an almost aconidial strain. Specifically, a significant reduction of approximately 100-fold in conidial production with respect to the wild type was observed (Figure 1A). Complementation of the *hbxA* deletion mutant with the *hbxA* wild-type allele restored normal conidiation. To gain insight into the *hbxA* mechanism that controls sporulation in *A. fumigatus* we examined the expression of the genes in the conidiation central regulatory pathway, *brlA*, *abaA*, and *wetA* [28]. Deletion of *hbxA* resulted in a significant reduction in the expression of all three genes compared to the wild type (Figure 2B, 2C and 2D).

Additionally, expression of the fluffy genes *flbB, flbD,* and *fluG* was also downregulated in the absence of *hbxA* (Figures 2E, 2F and 2G). Interestingly, microscopic observations indicated that conidial size in Δ*hbxA* was significantly larger than those formed by the wild type (Figure 3).

Absence of *hbxA* affects germination rates of *A. fumigatus* conidia.

To further examine the role of *hbxA* on conidia in *A. fumigatus*, an assay was performed to evaluate germination rates. As early as two hours post-inoculation, spores from the *hbxA* mutant started to produce germ tubes, while spores from wild type or complementation strain remained ungerminated (Figure 4A). Only after 6 hours did the wild-type spores begin to germinate but at that point more than 70% of the Δ*hbxA* spores were already germinated (Figure 4B).

Based on the rapid germination rate observed in the *hbxA* deletion mutant compared to

117 that of the control strains, biomass yields were also evaluated (Figure 4C). Cultures were grown
118 for 24 h and then lyophilized to determine the dry weight of the fungal biomass. Our results
119 indicated that the Δ*hbxA* strain generated almost two-fold the amount of biomass in comparison
120 to the wild type and complementation strain (Figure 4C).
121
122 **Deletion of *hbxA* causes an increase in sensitivity to the cell wall stressor SDS.**
123 Since absence of *hbxA* resulted in increased spore size and precocious germination, it is
124 possible that concomitant *hbxA*-dependent alterations in cell wall integrity could occur. To test
125 this hypothesis, the strains were grown on GMM containing different concentrations of SDS.
126 While all strains demonstrated colony growth reduction when exposed to all SDS concentrations
127 tested, the mutant exhibited greater sensitivity to that compound than the control, and was unable
128 to grow at a concentration of 0.015%, a condition that still allowed the growth of wild-type and
129 complementation colonies (Figure S2).
130
131 **Exposure to high concentration of sucrose partially rescues the *hbxA* conidiation defect.**
132 Osmotic stress has been shown to affect development of filamentous fungi, for example
133 increasing conidiation [29, 30]. We examine whether *hbxA* still influences conidiation when
134 exposed to osmotic stress. For most of osmotic stressors tested, no significant difference was
135 noticed in any of the strains except when Δ*hbxA* was grown in the presence of 1 M sucrose
136 (Figure S3). Under this condition the Δ*hbxA* strain produced more conidia than when grown on
137 GMM alone.
138 **Secondary metabolism in *A. fumigatus* is regulated by *hbxA*.**
139 In *A. flavus, hbx1* is a regulator of the production of numerous secondary metabolites,

including aflatoxin, aflatrem, and cyclopiazonic acid [26]. Similarly, we observed a broad regulatory scope of *hbxA* on secondary metabolism in *A. fumigatus* (Figure 5). Our results revealed that production of fumigaclavines, fumiquinazolines, and chaetomine, were detected in the Δ*hbxA*, but at a significantly lower levels than either the wild type or complementation strain.

Deletion of *hbxA* increases virulence of *A. fumigatus* in a murine model.

Due to the fact that *hbxA* plays an important role in regulating *A. fumigatus* development as well as its metabolome, we hypothesized that *hbxA* could also be relevant in virulence. To investigate this possibility, we used a neutropenic murine infection model. As shown in Figure 6, the Δ*hbxA* strain was significantly more virulent than the controls.

Discussion

In humans the primary route of *A. fumigatus* infections is through the inhalation of air-borne conidia that can eventually germinate in the lungs of a host. Investigation of genes that influence fungal development, as well as other aspects of *A. fumigatus* biology, could provide interesting targets to develop treatments against this opportunistic human pathogen. In the phylogenetically close and agriculturally important fungus *A. flavus,* the transcription regulatory gene *hbx1*, a homolog of *hbxA*, was found to regulate several aspects of morphological differentiation, including asexual development, as well as the synthesis of several secondary metabolites [26]. Homologs of *hbxA* have also been identified in other fungi beyond the *Aspergillus* genus, for example in *Magnaporthe oryzae* and in species of the genus *Fusarium.* In these species, while no connection of the possible role of *hbx1*/*hbxA* homologs with virulence or secondary metabolism was reported, the studies indicated a role in asexual development [31-33].

163 Our analysis of *A. fumigatus hbxA* demonstrated that this gene has indeed a conserved role in the
164 regulation of conidial production. Additionally, other aspect of conidial formation and function
165 were influenced by *hbxA* in this fungus. In the absence of *hbxA* conidia appear enlarged and
166 present a fast germination rate.

167 The mechanism of action of *hbxA* on conidiation includes the activation of the central
168 regulatory pathway, *brlA*, *abaA,* and *wetA* genes [28]. The expression of these three genes in this
169 signaling pathway is significantly reduced in the absence of *hbxA*. This decrease in expression
170 could lead to the observed reduction in conidial production in the *hbxA* mutant. In addition,
171 examination of the effect of *hbxA* on the expression of other genetic regulatory elements
172 upstream of the *brlA*-central regulatory pathway showed that *fluG,* a well-known developmental
173 regulator [34], is also *hbxA*-dependent. In addition, expression of *flbB* and *flbD* were also found
174 to be positively regulated by *hbxA.* FlbB has been previously shown to promote asexual
175 development in *A. fumigatus* [35]. FlbD has not been characterized in *A. fumigatus* [28], however
176 in *A. nidulans flbD* has been shown to also promote conidiation [36]. It is possible that HbxA
177 could regulate the expression of these "fluffy" developmental genes directly, which could
178 consequently affect the *brlA* pathway. In addition, since *flbD* expression is dependent on the
179 FlbB/FlbE protein complex [35,36], it is possible that *hbxA* could affect expression of *flbD*
180 indirectly by controlling *flbB* transcription. Interestingly, the presence of highly concentrated
181 sugars, particularly sucrose and fructose, resulted in a significant increase in conidial production
182 in the *hbxA* deletion mutant. Currently the mechanism that triggers conidiation under these
183 conditions is unknown.

184 In *A. fumigatus* and other fungi development and secondary metabolism are genetically
185 linked [22, 37, 38]. As in *A. flavus* [26], in our study we show that *hbxA* not only regulates

development in *A. fumigatus*, but also the production of multiple metabolites. Analysis of the *hbxA*-dependent metabolome revealed four different classes of compounds whose synthesis is under the influence of this regulator. Among them ergot alkaloids (fumigaclavine) and fumiquinazoline. These compounds present bioactive properties; fumigaclavine has been shown to affect the nervous systems of the host and induce apoptosis, while fumiquinazoline present cytotoxicity that inhibits neutrophils and aid *A. fumigatus* during infection [17, 39]. Fumigaclavines and fumiquinazolines, accumulate in asexual structures and their production is linked to *brlA* expression [16, 17, 20, 21]. Similarly, chaetominine is absent in the aconidial Δ*brlA* strain (misidentified as tryptoquivaline F) [20]. It is possible that *hbxA* regulatory role on the expression of genes involved in the synthesis of these compounds is indirect and mediated, at least in part, by its effect on *brlA.* In addition, the synthesis of chaetominine, a metabolite that is being tested to combat leukemia cells [40] is also controlled by *hbxA*. At the moment, it is unknown how this compound is produced or regulated in *A. fumigatus*. Chaetominine is structurally similar to fumiquinazolines, however, no evidence showing a link between their respective biosynthesis has been yet established. The present study reports a gene in *A. fumigatus* that controls production of chaetominine."

Due to the fact that *hbxA* is important in *A. fumigatus* morphogenesis and secondary metabolism we investigated whether this gene is relevant in virulence. To test this possibility, we used the neutropenic mouse infection model. Surprisingly, lack of *hbxA* did not attenuate virulence of this fungus, on the contrary it enhanced it, resulting in higher mortality rates in the group of animals infected by the deletion strain compared to the group inoculated with the wild-type strain. While production of secondary metabolites was hampered in the mutant, and conidial

size was larger, these traits did not reduce virulence in the mutant strain. It is possible that the premature germination observed in the *hbxA* mutant could have accounted for the increase in virulence, allowing the deletion mutant to rapidly establish itself in the host. This could lead to an accelerated and enhanced fungal infection in neutropenic mice compared to those infected with the wild type. The increased amounts of biomass yield produced by the Δ*hbxA* strain also suggest a greater capacity to colonize the host compared to the wild type. In addition, the detected increased sensitivity to SDS in the mutant could reflect premature changes in cell wall composition during the early spore germination process, which do not appear to be detrimental to infection.

In conclusion, we have established that the homeobox gene *hbxA* has a broad impact on the biology of *A. fumigatus*, affecting its development, secondary metabolism, and virulence. Specifically, we have shown that *hbxA* is necessary for normal asexual development, governing the expression the fluffy genes *fluG*, *flbB* and *flbD* as well as expression of those genes in the central developmental signaling pathway, *brlA*, *abaA* and *wetA*. With respect to the role of *hbxA* on *A. fumigatus* metabolome, our study revealed that production of several fungal alkaloids, as well as chaetominine are under its control. We also established that *hbxA* negatively affects pathogenicity, as lack of *hbxA* increases virulence, possibly through the advantage of early germination and greater biomass production. For this reason, an *hbxA* loss-of-function strategy would not the suitable against *A. fumigatus* infection. Future studies will focus on elucidating whether forced overexpression of this regulator would have the opposite effect on virulence and on the production of beneficial secondary metabolites such as chaetominine. Additional studies will also provide further insight into the identification of additional *hbxA*-dependent genetic elements involved in the regulation of conidiation, triggering of germination and synthesis of

232 fungal natural products.

233

234 ## Materials & Methods

235 ### Culture conditions

236 All strains used in this work are listed in Table 1. Strains were grown on glucose minimal
237 medium (GMM) at pH 6.5 in the dark at 37 °C, unless otherwise indicated. Agar at a
238 concentration of 1% was used for solid cultures. Stocks of each strain were maintained at -80 °C
239 in 30% glycerol. Spore inoculum was generated on GMM plus 1M sucrose and washed with
240 sterile water before use.

241 ### Strain Construction

242 *Generation of the deletion hbxA strain* (Δ*hbxA)*

243 To obtain the *hbxA* deletion strain, an *hbxA* deletion cassette was first generated by fusion
244 PCR as described by Szewczyk et al., [41]. Primers Afum_hbxA_P1 and Afum_hbxA_P2 were
245 used to PCR amplify the 5' UTR of the *hbxA* locus in the *A. fumigatus* genome, while
246 Afum_hbxA_P3 and Afum_hbxA_P4 primers were used to amplify the 3' UTR fragment. The
247 middle fragment containing the selection marker was PCR amplified from plasmid pPG28 [42]
248 using primers Afum_hbxA_P5 and Afum_hbxA_P6. The marker used was *pyrG* from
249 *Aspergillus parasiticus.* The three fragments were then fused by PCR using primers
250 Afum_hbxA_P7 and Afum_hbxA_P8. All primers utilized in this study are listed in Table 2. The
251 fused PCR product was transformed into *A fumigatus* CEA10 (*pyrG-*,
252 *ptrA*-) by a polyethylene glycol-mediated transformation as previously described (Cary et al.,
253 2017). Transformants were selected on half strength PDA without uracil. Potassium chloride (0.6

M) was used as osmotic stabilizer in the regeneration medium. Transformants were confirmed by diagnostic PCR with primers Afum_hbxA_P0 and Apara_pyrG_R. A selected *hbxA* deletion transformant, TTRS8, was used in this study.

Generation of the complementation hbx1 strain (Com)

To generate the complementation strain a two-fragment fusion PCR method was utilized joining the *hbxA* locus and the selection marker gene *ptrA* from *Aspergillus oryzae*. First, the *hbxA* locus was amplified from the *A. fumigatus* genomic DNA, using primers Afum_hbxA_Com_P1 & Afum_hbxA_Com_P2, while the *ptrA* marker was amplified from the plasmid pPTRI (TakaraBIO, Mountain View, CA, USA) with primers Afum_hbxA_Com_P3 & Afum_hbxA_Com_P4. The two PCR products were then fused in a similar manner as described above using primers Afum_hbxA_Com_P1 & Afum_hbxA_Com_P4. The fusion cassette was transformed into the Δ*hbxA* strain TTRS8. Transformants were selected on Czapek Dox (CZ, Difco, Franklin Lakes, New Jersey, USA) medium containing 1 µg/ml of pyrithiamine. Confirmation of the reinsertion of *hbxA* in the transformants was carried out by diagnostic PCR with primers Afum_hbxA_qPCR_F and R_ptrA_Check. A selected *hbxA* complementation strain, TTRS12 was used in this study. Pertinent genotypes of all strains are listed in Table 1.

Morphological Analysis

Colony growth

Wild type, Δ*hbxA*, and complementation strains were point-inoculated on GMM. Colony diameter was measured after 5 days of incubation at 37 °C. The experiment was carried out with three replicates.

Conidial production

To assess whether *hbxA* regulates conidiation in *A. fumigatus*, 10^6 spores/ml of each, wild type, Δ*hbxA*, and complementation strains, were inoculated into 25 ml of liquid GMM. Cultures were grown under stationary conditions allowing an air interphase to promote development. Cores (7 mm diameter) were collected from the mycelial mats to quantify conidia after 48 h and 72 h of incubation at 37 °C. The cores were homogenized in water and spores were counted under a Nikon Eclipse E-400 bright-field microscope (Nikon Inc., Melville, NY, USA microscope) using a hemocytometer (Hausser Scientific, Horsham, PA). Experiments were performed in triplicate.

Effect of osmotic stress on hbxA-dependent growth and conidiation

To examine the possible role of *hbxA* on osmotic stress resistance, the wild type, Δ*hbxA*, and complementation strains, were point-inoculated on solid GMM and GMM plus either 0.6 M KCl, 1 M sucrose, 0.7 M NaCl, or 1.2 M sorbitol. Cultures were incubated in the dark at 37 °C for 7 days. Colonies growth was assessed as colony diameter. Under these conditions' conidia were also quantified. Cores were collected from the colonies and spores were counted as described above.

Cell wall stress test

To assess whether *hbxA* influences the sensitivity to cell wall stress, the wild type, Δ*hbxA* and complementation strains were point-inoculated on solid GMM containing 0%, 0.01%, 0.015% or 0.02% SDS. The experiment was performed in triplicate. Cultures were incubated at 37º C for 72 h.

Germination assay

Flasks with 50 ml of liquid GMM were inoculated with conidia (10^6 spores/ml) of wild type, Δ*hbxA*, and complementation strains. Every two hours post-inoculation, five hundred

microliters of culture was collected from each flask for spore quantification under the microscope using a hemocytometer. Micrographs were taken with a Nikon Eclipse E-600 microscope with total magnification of 400X. The experiment was performed in triplicate.

Determination of dry weight

Cultures of 50 ml of liquid GMM inoculated with 10^6 spores/ml of wild type, Δ*hbxA*, and complementation strains were grown in a shaker incubator at 250 rpm at 37º C. After 24 h of incubation the total content was transferred to a 50 mL Falcon tube and centrifuged at 3500 rpm for 5 minutes. The supernatant was removed, the biomass was lyophilized for 48h and then weighed.

Gene Expression Analysis

Petri dishes containing 25 ml of liquid GMM were inoculated with conidia (10^6 spores/ml) of *A. fumigatus* CEA10 WT, Δ*hbxA*, and complementation strains. Cultures were incubated in stationary conditions at 37 °C in the dark. Total RNA was extracted from lyophilized mycelial samples using TRIsure (Bioline, Taunton, MA, USA) reagent according to the manufacturer instructions. cDNA was synthesized with Moloney murine leukemia virus (MMLV) reverse transcriptase (Promega, Madison, WI, USA). qRT-PCR was performed with the Applied Biosystems 7000 Real-Time PCR System using SYBR green dye for fluorescence detection. cDNA was normalized to *A. fumigatus* 18S ribosomal gene expression, and the relative expression levels were calculated using the $2^{-\Delta\Delta CT}$ [44]. Primer pairs used are indicated in Table 2.

Liquid chromatography and mass spectrometry analysis

Sample analysis was performed using HPLC coupled to an LTQ Orbitrap XL high-resolution

mass spectrometer (Thermo Fisher Scientific, Les Ulis, France). Extracts were resuspended in 500 µl of acetonitrile/water (50:50, v/v) and then mixed 250 µl of acetonitrile and 10 µl of this suspension were injected into a reversed-phase (150 mm × 2.0 mm) 5 µm Luna C18 column (Phenomenex, Torrance, CA, U.S.A.) operated at a flow rate of 0.2 mL/min. A gradient program was performed with 0.1% formic acid (phase A) and 100% acetonitrile (phase B) with the following elution gradient: 0 min 20% B, 30 min 50% B, from 35 to 45 min 90% B, from 50 to 60 min 20% B. HRMS acquisitions were achieved with electrospray ionization (ESI) in the positive and negative modes as follows: spray voltage +4.5 kV, capillary temperature 350°C, sheath gas (N_2) flow rate 40 au (arbitrary units), auxiliary gas (N_2) flow rate 6 au in the positive mode, and spray voltage −3.7 kV, capillary temperature 350°C, sheath gas (N_2) flow rate 30 au, auxiliary gas (N_2) flow rate 10 au in the negative mode. Full MS spectra were acquired at a resolution of 60,000 with a range of mass-to-charge ratio (m/z) set to 50−800. The chaetominine standard was purchased from BioAustralis Fine chemicals (Smithfield, Australia).

Pathogenicity analysis

Pathogenicity studies using a neutropenic mouse model were carried out as previously described [45], with minor modifications. Briefly, six-week old female, outbred ICR Swiss mice, weighing approximately 25 g were used for this experiment. Fifty mice divided into 5 separate groups were used, each group contained 10 mice. Animals were rendered neutropenic by intraperitoneal injection of cyclophosphamide (150 mg/kg of body weight) on days -4, -1 and 3 days post infection and Kenalog (40 mg/kg) on the day of infection. The immunosuppressed mice were infected with fungal spores of *A. fumigatus* CEA10 wild type, Δ*hbxA*, and complementation strains. Sedated mice (10 mice per strain) were infected by nasal instillation of $2x10^6$ spores/40 µl of PBS. Post infection mice were observed three times daily. Mice that survived to day eight were euthanized.

This study was carried out in strict accordance with the Guide for the Care and Use of

Laboratory Animals of the National Research Council. The protocol was approved by the Institutional Animal Care and Use Committee of Northern Illinois University (Permit #12–0006). All efforts were made to minimize suffering. Humane euthanasia by CO_2 inhalation was performed when mice met criteria indicating a moribund state; these endpoints include behaviors of unresponsiveness to tactile stimuli, inactivity, lethargy, staggering, anorexia and/or clinical signs of bleeding from the nose or mouth, labored breathing, agonal respirations, purulent exudate from eyes or nose, abnormally ruffled fur, or greater than 20% weight loss. The method of euthanasia by CO_2 inhalation is consistent with recommendations of the Panel on Euthanasia of the American Veterinary Medical Association.

Statistical Analysis

Statistical analysis was applied to analyze all of the quantitative data in this study utilizing ANOVA (analysis of variance), in conjunction with a Tukey's multiple comparison test using a p-value of $p < 0.05$ for samples that are determined to be significantly different. The exception was for pathogenicity assays in which a Kaplan-Meyer Survival test was applied.

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500

501 **Table 1**

502 **List of strains used in this study**

Strain	Genotype	Source
CEA10	Wild type	Gift from Robert Cramer
CEA17	*pyrG1*	Gift from Robert Cramer
TTRS8	*pyrG1*, Δ*hbxA*::*pyrG*$^{A.parasiticus}$	This study
TTRS12	*pyrG1*, Δ*hbxA*::*pyrG*$^{A.parasiticus}$,*hbxA*::*ptrA*$^{A.oryzae}$	This study

503

504 **Table 2**

505 **Primers used in this study**

Primers	Sequence
Afum_hbxA_P0	GTGGTGACAGTGGTGGTTTCCC
Afum_hbxA_P1	GGTGCTTAGTTCCCTGGATGGACA
Afum_hbxA_P2	AAGGGACGGCGGAGAAGAAG
Afum_hbxA_P3	GTAGTGGTAGGCAGGAGGCATG
Afum_hbxA_P4	TGCTTGTAGTCACCGATCACCATTCC
Afum_hbxA_P5	CTTCTTCTCCGCCGTCCCTTGGATCCTATGGATCTCAGAACAATATACC
Afum_hbxA_P6	CATGCCTCCTGCCTACCACTACGTCGACATCACCCTTACCCA
Afum_hbxA_P7	CAAGACAGAATGACTGCCCAAACTGG
Afum_hbxA_P8	CAGTCACCCCATTCCACAGCT
Apara_pyrG_R	CAGGAGCAGCATAAATTCCACGACC
Afum_hbxA_Com_P1	CAAGACAGAATGACTGCCCAAACTGG
Afum_hbxA_Com_P2	GGCTCATCGTCACCCCATTTTGTTAAGAAGCGTTGCCATTGCGTGA

Afum_hbxA_Com_P3	TCACGCAATGGCAACGCTTCTTAACAAAATGGGGTGACGATGAGCC
Afum_hbxA_Com_P4	TCAATGGGCAATTGATTACGGGATCC
R_ptrA Check	CAGCTGCCATCTACGAACCCAC
AFUM 18SqPCR F	TAGTCGGGGGCGTCAGTATTCAGC
AFUM 18S qPCR R	GTAAGGTGCCGAGCGGGTCATCAT
AFUM hbxA qPCR F	GGAGGAGACCGATAAAGCCAACGA
AFUM hbxA qPCR R	GCCGTCATTCCGATCCTGCTC
AFUM brlA qPCR F	TGCACCAATATCCGCCAATGC
AFUM brlA qPCR R	CGTGTAGGAAGGAGGAGGGGTTACC
AFUM abaA qPCR F	CCGCCGCAGGAGACTAGTCAG
AFUM abaA qPCR R	CTGTCGTGAACGCTAACGCCG
AFUM wetA qPCR F	TTGACTCGCTGTCAAGTGATTGTGG
AFUM wetA qPCR R	TGGTGGATTTGTGGTGGGGAGTT
AFUM flbB qPCR F	GCCTTGACACGACGACAGGAAC
AFUM flbB qPCR R	CTGAGCGTCGTTCTGCCCT
AFUM flbD qPCR F	GGGAGCGATTCCACCAGAACC
AFUM flbD qPCR R	ATGGGGCTTCGACTCGGC
AFUM fluG qPCR F	GGGGTAGCTCTACAGAATGCGACT
AFUM fluG qPCR R	CCATTGGTACGGCTCGATGTCC

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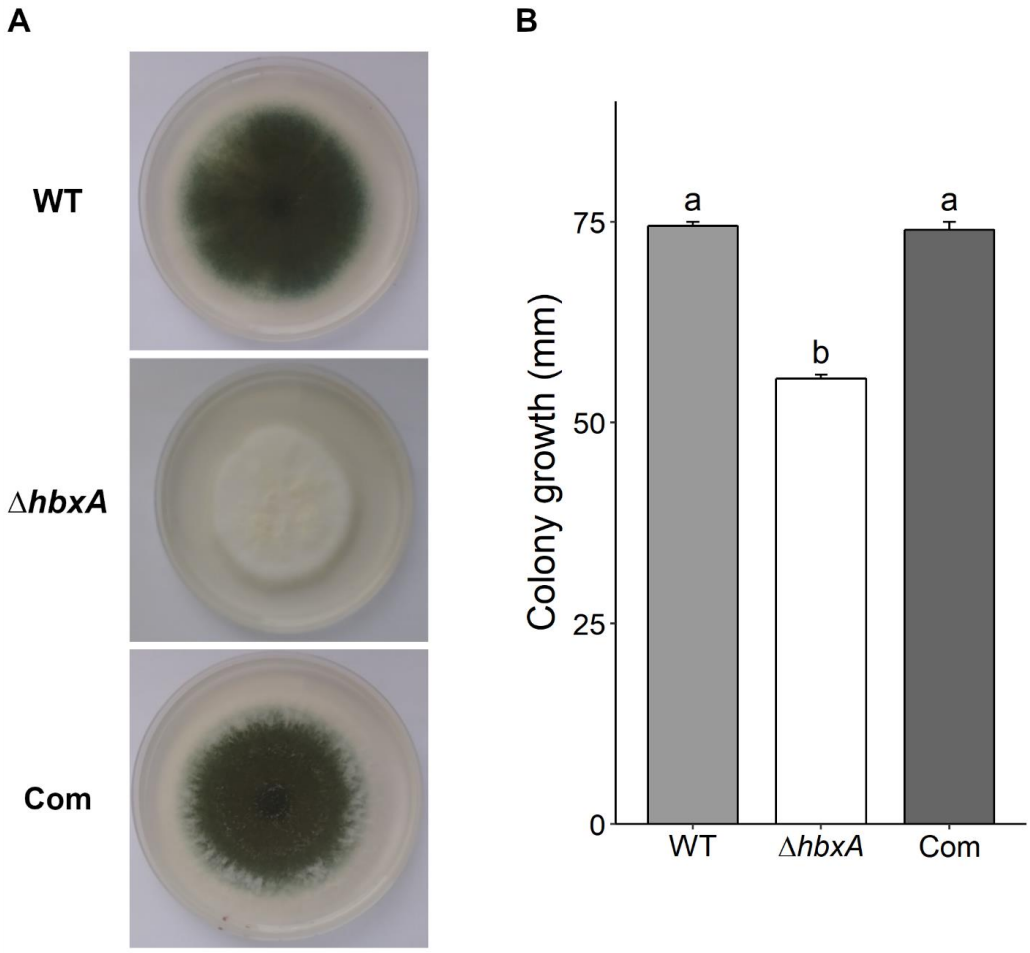


510

511 **Figure 1- *hbxA* is required for normal colony development.**

512 A CEA10 wild type (WT), Δ*hbxA,* and complementation (Com) strains were point-inoculated on

513 solid GMM and allowed to grow in the dark at 37 °C. (A) Images of the colonies after 5 days of

514 growth. (B) Quantification of colony growth measured as colony diameter. Error bars are used

515 to indicate standard error. Different letters on the columns indicate values that are statistically

516 different ($p < 0.05$) as determined by ANOVA with the Tukey test comparison. This experiment

was performed three times with three biological replicates in each experiment.

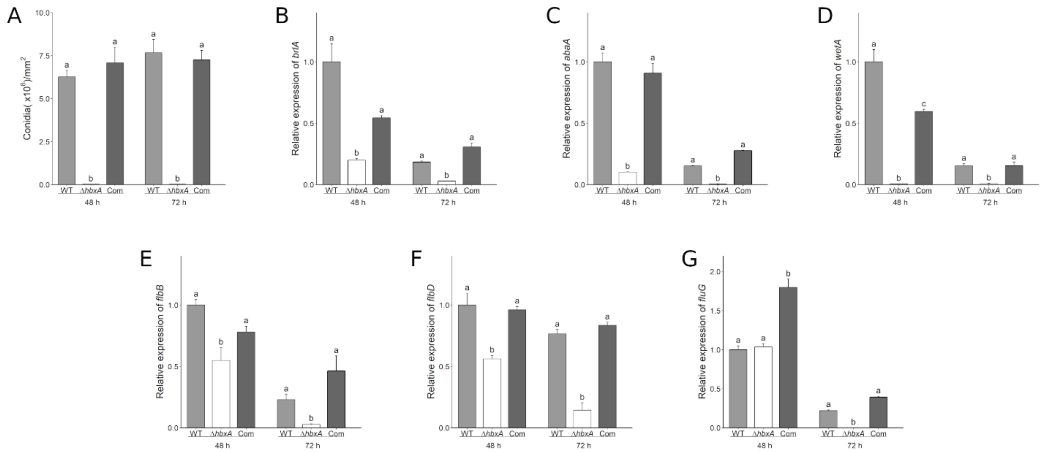
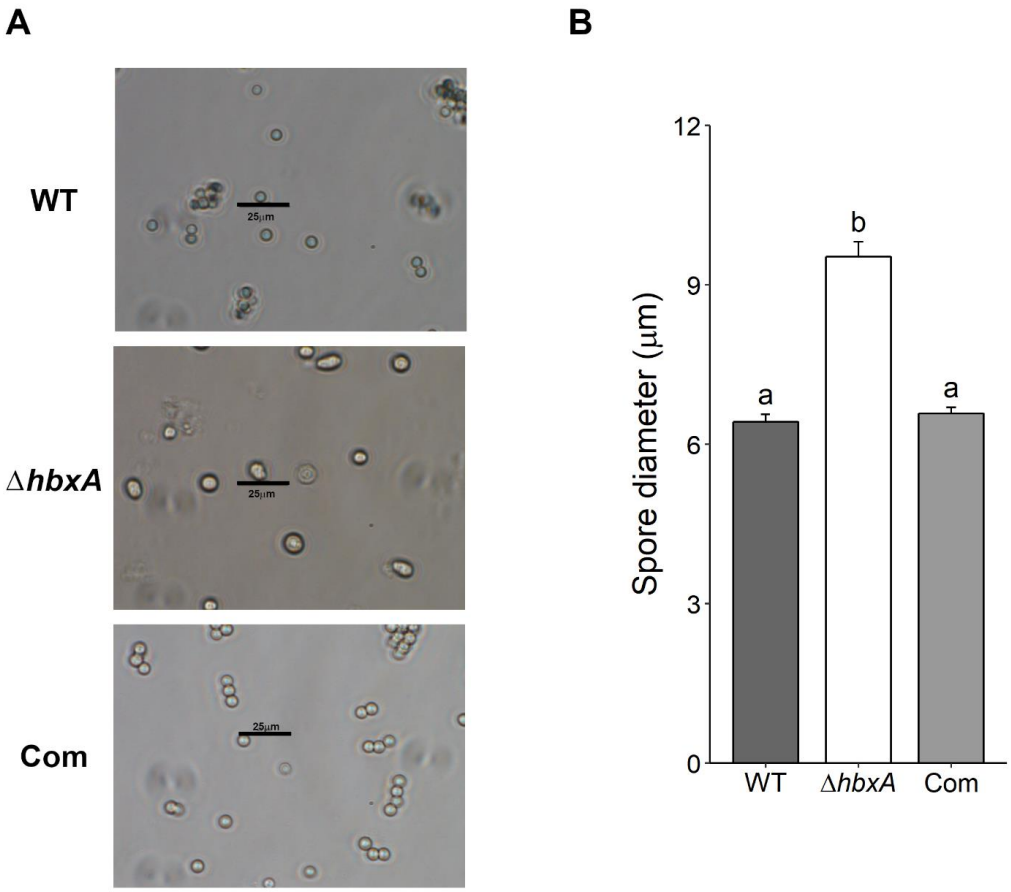


Figure 2 – Asexual development is strongly regulated by *hbxA* in *A. fumigatus.*

Wild type (WT), Δ*hbxA,* and complementation (Com) strains were grown in liquid GMM stationary cultures for 72 h. Cores from the mycelial mats were collected at 48 h and 72 h, and conidia were quantified (A). RNA was also extracted from mycelia to perform qRT-PCR. Relative gene expression of *brlA*(B), *abaA*(C), *wetA*(D), *flbB*(E), *flbD*(F), and *fluG*(G) is shown. All values were normalized to the wild-type 48 h samples. Error bars indicate standard error. Different letters on the columns indicate values that are statistically different ($p < 0.05$) as determined by ANOVA with the Tukey test comparison. This experiment was performed two times with three biological replicates in each experiment. For qRT-PCR technical replicates were also run for each biological replicate.



533

534 **Figure 3 –Size of conidia is influenced by *hbxA*.**

535 Wild type (WT), Δ*hbxA,* and complementation (Com) strains were grown on GMM and spores
536 were collected after 72 h. Samples were observed under the microscope, and spore diameter was
537 measured. (A) Micrographs of conidia. (B) Measurements of spore diameter from 40 spores of
538 each strain. Error bars indicate standard error. Different letters on the columns indicate values
539 that are statistically different ($p < 0.05$) as determined by ANOVA with the Tukey test

540 comparison. This experiment was performed three times with three biological replicates in each

541 experiment.

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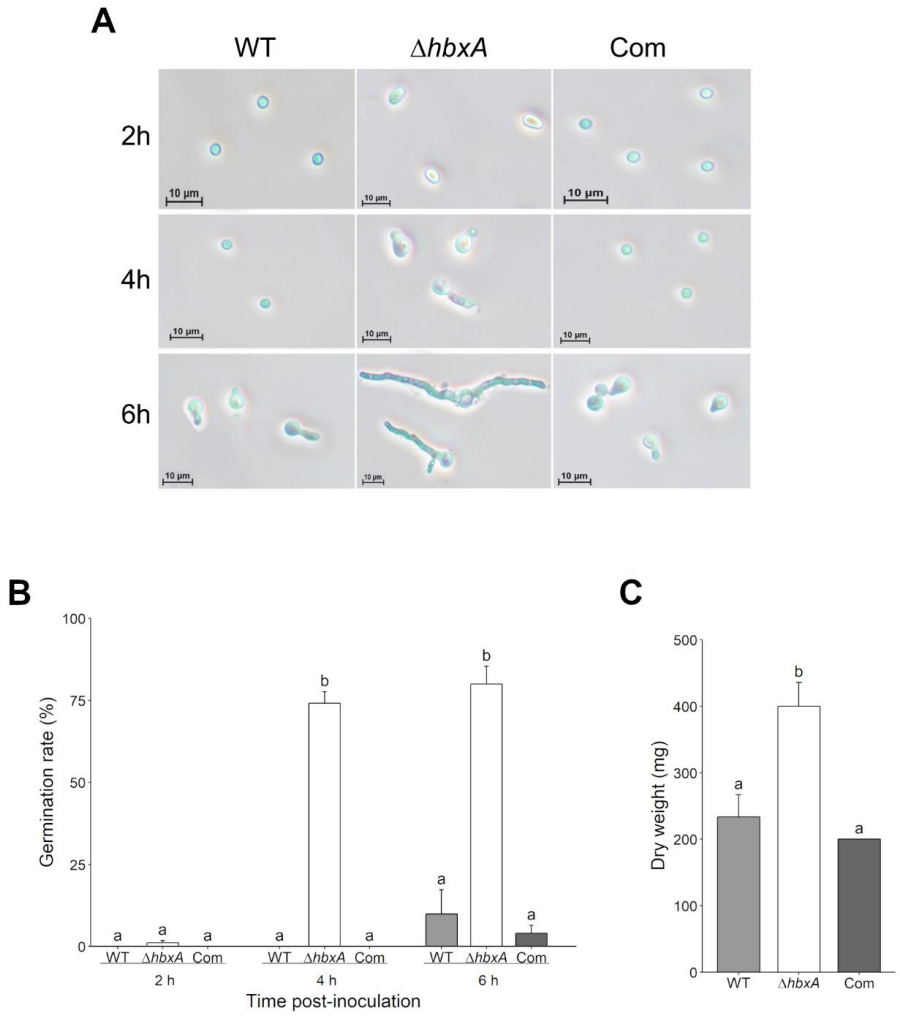

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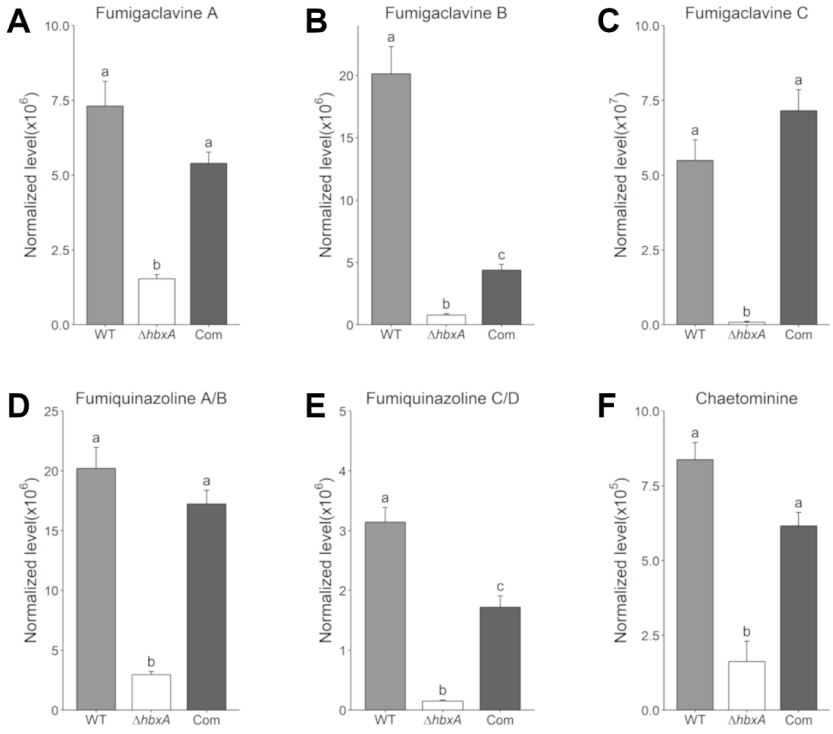
544

545 **Figure 4 - Absence of *hbxA* results in earlier spore germination in *A. fumigatus***

546 Liquid GMM cultures of wild type (WT), Δ*hbxA,* and complementation (Com) strains were
547 inoculated with 10^6 spores/ml. Every 2 h post-inoculation an aliquot of 500 µl of culture was
548 collected to observe conidial germination. (A) Micrographs of germinating conidia at different
549 time points. (B) Percentage of germinated conidia. (C) Amount of biomass produced by each
550 strain after 24 h in liquid shaking conditions. Error bars are used to indicate standard error.
551 Different letters on the columns indicate values that are statistically different ($p < 0.05$) as
552 determined by ANOVA with the Tukey test comparison. This experiment was performed four
553 times with three biological replicates in each experiment.



554

555 **Figure 5 – *hbxA* regulates the production of multiple secondary metabolites in *A. fumigatus***
556 Spores of *A. fumigatus* wild type (WT), deletion (Δ*hbxA*) and complementation (com) strain

557 were inoculated in liquid YES medium. Cultures were incubated at 37°C, and supernatant was
558 collected after 7 days for secondary metabolite extraction and analysis. (A, B, & C) Analysis of
559 ergot alkaloids fumigaclavine A, B and C. (D & E) Analysis of fumiquinazoline A/B & C/D. (F)
560 Analysis of chaetominine. Error bars are used to indicate standard error. Different letters on the
561 columns indicate values that are statistically different ($p < 0.05$) as determined by ANOVA with
562 the Tukey test comparison. This experiment was performed two times with four biological
563 replicates in each experiment.
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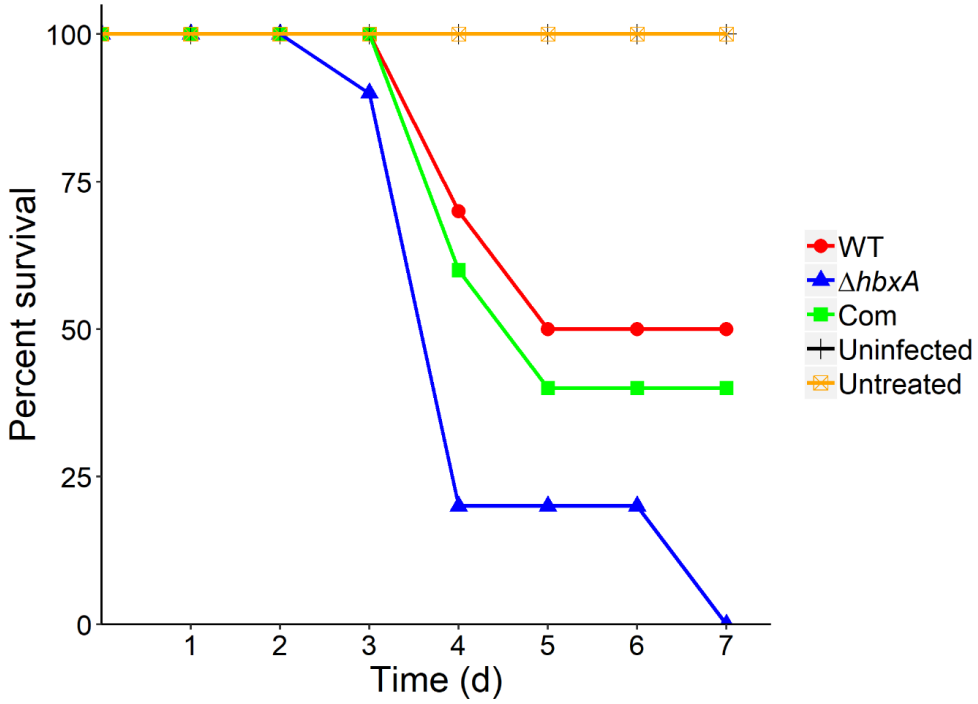

565

566 **Figure 6 – *hbxA* negatively regulates virulence in a mouse model**

567 Six-week old mice were rendered neutropenic by administration of cyclophosphamide and
568 Kenalog-10 treatments. Fifty mice divided into 5 separate groups were used, each group

569 contained 10 mice. Mice were infected with $2x10^6$ conidia/mouse of *A. fumigatus* wild type
570 (WT), deletion (Δ*hbxA*) and complementation (com) strain and monitored daily for a total of
571 seven days. Two controls that did not received fungal spores were included in this analysis: a
572 group that was rendered neutropenic (uninfected) and another group not treated with
573 cyclophosphamide or Kenalog-10 (untreated).Statistical analysis of survival was carried out by a
574 Kaplan-Meyer pairwise comparison using a long rank test.