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

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

3

4           Timothy Satterlee<sup>1</sup>, Binita Nepal<sup>1</sup>, Sophie Lorber<sup>2</sup>, Olivier Puel<sup>2</sup>, and Ana M. Calvo<sup>1\*</sup>

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14

15       **Abstract**

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

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

30

### 31 **Importance of work**

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

44

### 45 **Introduction**

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

48 particularly susceptible 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.

## 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  $\Delta hbxA$  strain, while the complementation strain showed a restoration of *hbxA* expression

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

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

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

108

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

110 To further examine the role of *hbxA* on conidia in *A. fumigatus*, an assay was performed  
111 to evaluate germination rates. As early as two hours post-inoculation, spores from the *hbxA*  
112 mutant started to produce germ tubes, while spores from wild type or complementation strain  
113 remained ungerminated (Figure 4A). Only after 6 hours did the wild-type spores begin to  
114 germinate but at that point more than 70% of the  $\Delta hbxA$  spores were already germinated (Figure  
115 4B).

116 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  $\Delta hbxA$  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  $\Delta hbxA$  was grown in the presence of 1 M sucrose  
136 (Figure S3). Under this condition the  $\Delta hbxA$  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*, *hbxA* is a regulator of the production of numerous secondary metabolites,

140 including aflatoxin, aflatrem, and cyclopiazonic acid [26]. Similarly, we observed a broad  
141 regulatory scope of *hbxA* on secondary metabolism in *A. fumigatus* (Figure 5). Our results  
142 revealed that production of fumigaclavines, fumiquinazolines, and chaetomine, were detected in  
143 the  $\Delta hbxA$ , but at a significantly lower levels than either the wild type or complementation strain.  
144

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

146 Due to the fact that *hbxA* plays an important role in regulating *A. fumigatus* development  
147 as well as its metabolome, we hypothesized that *hbxA* could also be relevant in virulence. To  
148 investigate this possibility, we used a neutropenic murine infection model. As shown in Figure 6,  
149 the  $\Delta hbxA$  strain was significantly more virulent than the controls.  
150

#### 151 **Discussion**

152 In humans the primary route of *A. fumigatus* infections is through the inhalation of air-  
153 borne conidia that can eventually germinate in the lungs of a host. Investigation of genes that  
154 influence fungal development, as well as other aspects of *A. fumigatus* biology, could provide  
155 interesting targets to develop treatments against this opportunistic human pathogen. In the  
156 phylogenetically close and agriculturally important fungus *A. flavus*, the transcription regulatory  
157 gene *hbx1*, a homolog of *hbxA*, was found to regulate several aspects of morphological  
158 differentiation, including asexual development, as well as the synthesis of several secondary  
159 metabolites [26]. Homologs of *hbxA* have also been identified in other fungi beyond the  
160 *Aspergillus* genus, for example in *Magnaporthe oryzae* and in species of the genus *Fusarium*. In  
161 these species, while no connection of the possible role of *hbx1/hbxA* homologs with virulence or  
162 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

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

202

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

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

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

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

254 M) was used as osmotic stabilizer in the regeneration medium. Transformants were confirmed by  
255 diagnostic PCR with primers Afum\_hbxA\_P0 and Apara\_pyrG\_R. A selected *hbxA* deletion  
256 transformant, TTRS8, was used in this study.

#### 257 *Generation of the complementation hbxA strain (Com)*

258 To generate the complementation strain a two-fragment fusion PCR method was utilized  
259 joining the *hbxA* locus and the selection marker gene *ptrA* from *Aspergillus oryzae*. First, the  
260 *hbxA* locus was amplified from the *A. fumigatus* genomic DNA, using primers  
261 Afum\_hbxA\_Com\_P1 & Afum\_hbxA\_Com\_P2, while the *ptrA* marker was amplified from the  
262 plasmid pPTRI (TakaraBIO, Mountain View, CA, USA) with primers Afum\_hbxA\_Com\_P3 &  
263 Afum\_hbxA\_Com\_P4. The two PCR products were then fused in a similar manner as described  
264 above using primers Afum\_hbxA\_Com\_P1 & Afum\_hbxA\_Com\_P4. The fusion cassette was  
265 transformed into the  $\Delta hbxA$  strain TTRS8. Transformants were selected on Czapek Dox (CZ,  
266 Difco, Franklin Lakes, New Jersey, USA) medium containing 1  $\mu\text{g/ml}$  of pyrithiamine.  
267 Confirmation of the reinsertion of *hbxA* in the transformants was carried out by diagnostic PCR  
268 with primers Afum\_hbxA\_qPCR\_F and R\_ptrA\_Check. A selected *hbxA* complementation  
269 strain, TTRS12 was used in this study. Pertinent genotypes of all strains are listed in Table 1.

270

#### 271 **Morphological Analysis**

##### 272 *Colony growth*

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

##### 276 *Conidial production*

277 To assess whether *hbxA* regulates conidiation in *A. fumigatus*,  $10^6$  spores/ml of each, wild  
278 type,  $\Delta hbxA$ , and complementation strains, were inoculated into 25 ml of liquid GMM. Cultures  
279 were grown under stationary conditions allowing an air interphase to promote development.  
280 Cores (7 mm diameter) were collected from the mycelial mats to quantify conidia after 48 h and  
281 72 h of incubation at 37 °C. The cores were homogenized in water and spores were counted  
282 under a Nikon Eclipse E-400 bright-field microscope (Nikon Inc., Melville, NY, USA  
283 microscope) using a hemocytometer (Hausser Scientific, Horsham, PA). Experiments were  
284 performed in triplicate.

285 *Effect of osmotic stress on hbxA-dependent growth and conidiation*

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

292 *Cell wall stress test*

293 To assess whether *hbxA* influences the sensitivity to cell wall stress, the wild type,  $\Delta hbxA$   
294 and complementation strains were point-inoculated on solid GMM containing 0%, 0.01%,  
295 0.015% or 0.02% SDS. The experiment was performed in triplicate. Cultures were incubated at  
296 37° C for 72 h.

297 *Germination assay*

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

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

### 303 *Determination of dry weight*

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

309

### 310 **Gene Expression Analysis**

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

### 321 **Liquid chromatography and mass spectrometry analysis**

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

323 mass spectrometer (Thermo Fisher Scientific, Les Ulis, France). Extracts were resuspended in 500  $\mu$ l of  
324 acetonitrile/water (50:50, v/v) and then mixed 250  $\mu$ l of acetonitrile and 10  $\mu$ l of this suspension were  
325 injected into a reversed-phase (150 mm  $\times$  2.0 mm) 5  $\mu$ m Luna C18 column (Phenomenex, Torrance, CA,  
326 U.S.A.) operated at a flow rate of 0.2 mL/min. A gradient program was performed with 0.1% formic acid  
327 (phase A) and 100% acetonitrile (phase B) with the following elution gradient: 0 min 20% B, 30 min 50%  
328 B, from 35 to 45 min 90% B, from 50 to 60 min 20% B. HRMS acquisitions were achieved with  
329 electrospray ionization (ESI) in the positive and negative modes as follows: spray voltage +4.5 kV,  
330 capillary temperature 350°C, sheath gas (N<sub>2</sub>) flow rate 40 au (arbitrary units), auxiliary gas (N<sub>2</sub>) flow rate  
331 6 au in the positive mode, and spray voltage -3.7 kV, capillary temperature 350°C, sheath gas (N<sub>2</sub>) flow  
332 rate 30 au, auxiliary gas (N<sub>2</sub>) flow rate 10 au in the negative mode. Full MS spectra were acquired at a  
333 resolution of 60,000 with a range of mass-to-charge ratio (m/z) set to 50–800. The chaetominine standard  
334 was purchased from BioAustralis Fine chemicals (Smithfield, Australia).

### 335 **Pathogenicity analysis**

336 Pathogenicity studies using a neutropenic mouse model were carried out as previously  
337 described [45], with minor modifications. Briefly, six-week old female, outbred ICR Swiss mice,  
338 weighing approximately 25 g were used for this experiment. Fifty mice divided into 5 separate  
339 groups were used, each group contained 10 mice. Animals were rendered neutropenic by  
340 intraperitoneal injection of cyclophosphamide (150 mg/kg of body weight) on days -4, -1 and 3  
341 days post infection and Kenalog (40 mg/kg) on the day of infection. The immunosuppressed  
342 mice were infected with fungal spores of *A. fumigatus* CEA10 wild type,  $\Delta$ *hbxA*, and  
343 complementation strains. Sedated mice (10 mice per strain) were infected by nasal instillation of  
344  $2 \times 10^6$  spores/40  $\mu$ l of PBS. Post infection mice were observed three times daily. Mice that  
345 survived to day eight were euthanized.

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



347 Laboratory Animals of the National Research Council. The protocol was approved by the  
348 Institutional Animal Care and Use Committee of Northern Illinois University (Permit #12-0006).  
349 All efforts were made to minimize suffering. Humane euthanasia by CO<sub>2</sub> inhalation was  
350 performed when mice met criteria indicating a moribund state; these endpoints include behaviors  
351 of unresponsiveness to tactile stimuli, inactivity, lethargy, staggering, anorexia and/or clinical  
352 signs of bleeding from the nose or mouth, labored breathing, agonal respirations, purulent  
353 exudate from eyes or nose, abnormally ruffled fur, or greater than 20% weight loss. The method  
354 of euthanasia by CO<sub>2</sub> inhalation is consistent with recommendations of the Panel on Euthanasia  
355 of the American Veterinary Medical Association.

356

### 357 **Statistical Analysis**

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

362

### 363 **Acknowledgments**

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365

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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	<i>pyrG1</i>	Gift from Robert Cramer
TTRS8	<i>pyrG1</i> , $\Delta$ <i>hbxA::pyrG<sup>A.parasiticus</sup></i>	This study
TTRS12	<i>pyrG1</i> , $\Delta$ <i>hbxA::pyrG<sup>A.parasiticus</sup>, <i>hbxA::ptrA<sup>A.oryzae</sup></i></i>	This study

503

504

**Table 2**

505

**Primers used in this study**

Primers	Sequence
Afum_hbxA_P0	GTGGTGACAGTGGTGGTTCC
Afum_hbxA_P1	GGTGCTTAGTTCCTGGATGGACA
Afum_hbxA_P2	AAGGGACGGCGGAGAAGAAG
Afum_hbxA_P3	GTAGTGGTAGGCAGGAGGCATG
Afum_hbxA_P4	TGCTTGTAGTCACCGATCACCATTCC
Afum_hbxA_P5	CTTCTTCTCCGCGTCCCTTGGATCCTATGGATCTCAGAACAATATACC
Afum_hbxA_P6	CATGCCTCTGCCTACCACTACGTCGACATCACCCTTACCCA
Afum_hbxA_P7	CAAGACAGAATGACTGCCCAAAGTGG
Afum_hbxA_P8	CAGTACCCCATTCACAGCT
Apara_pyrG_R	CAGGAGCAGCATAAATTCACGACC
Afum_hbxA_Com_P1	CAAGACAGAATGACTGCCCAAAGTGG
Afum_hbxA_Com_P2	GGCTCATCGTCACCCATTTTGTAAAGAAGCGTTGCCATTGCGTGA

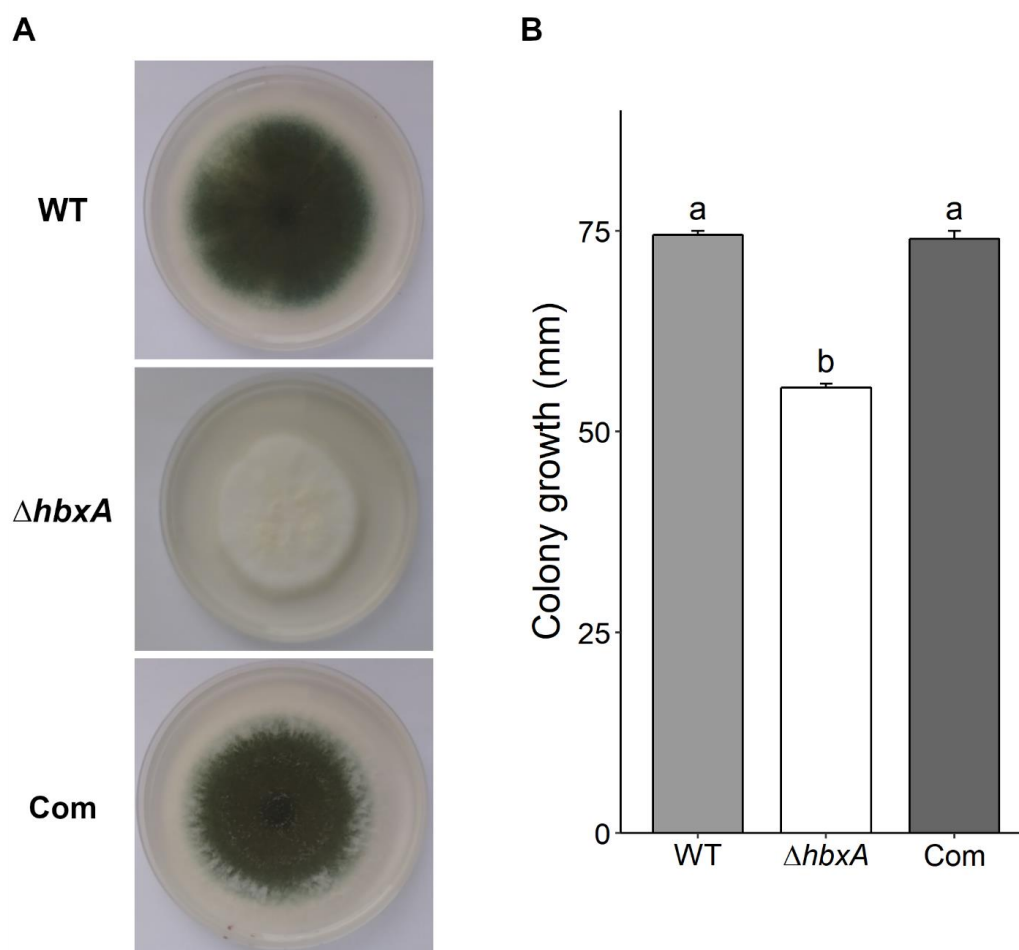


Afum_hbxA_Com_P3	TCACGCAATGGCAACGCTTCTTAACAAAATGGGGTGACGATGAGCC
Afum_hbxA_Com_P4	TCAATGGGCAATTGATTACGGGATCC
R_ptrA Check	CAGCTGCCATCTACGAACCCAC
AFUM 18SqPCR F	TAGTCGGGGGCGTCAGTATTCAGC
AFUM 18S qPCR R	GTAAGGTGCCGAGCGGGTCATCAT
AFUM hbxA qPCR F	GGAGGAGACCGATAAAGCCAACGA
AFUM hbxA qPCR R	GCCGTCATTCCGATCCTGCTC
AFUM brlA qPCR F	TGCACCAATATCCGCAATGC
AFUM brlA qPCR R	CGTGTAGGAAGGAGGAGGGGTTACC
AFUM abaA qPCR F	CCGCCGAGGAGACTAGTCAG
AFUM abaA qPCR R	CTGTCGTGAACGCTAACGCCG
AFUM wetA qPCR F	TTGACTCGCTGTCAAGTGATTGTGG
AFUM wetA qPCR R	TGGTGGATTTGGTGGGGAGTT
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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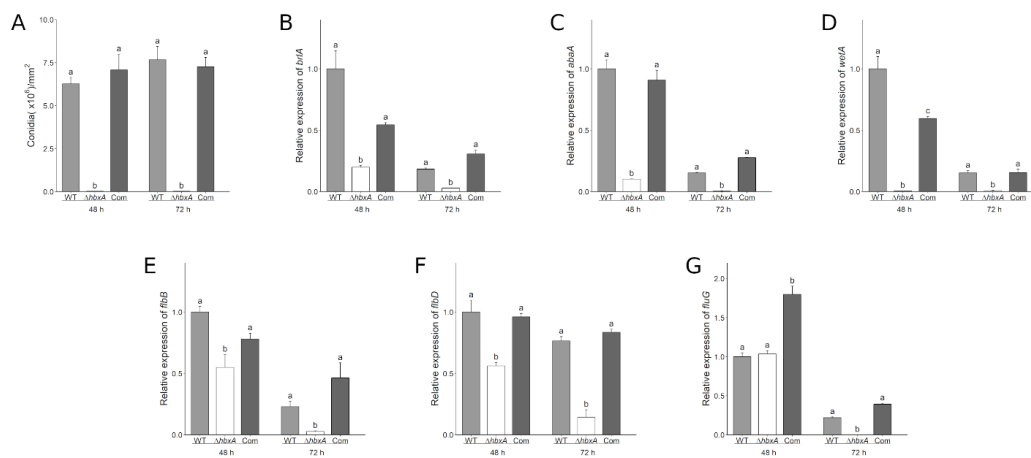
510

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

512 A CEA10 wild type (WT),  $\Delta hbxA$ , 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

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

518



519

520

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

522 Wild type (WT),  $\Delta hbxA$ , and complementation (Com) strains were grown in liquid GMM

523 stationary cultures for 72 h. Cores from the mycelial mats were collected at 48 h and 72 h, and

524 conidia were quantified (A). RNA was also extracted from mycelia to perform qRT-PCR.

525 Relative gene expression of *brlA*(B), *abaA*(C), *wetA*(D), *flbB*(E), *flbD*(F), and *fluG*(G) is shown.

526 All values were normalized to the wild-type 48 h samples. Error bars indicate standard error.

527 Different letters on the columns indicate values that are statistically different ( $p < 0.05$ ) as

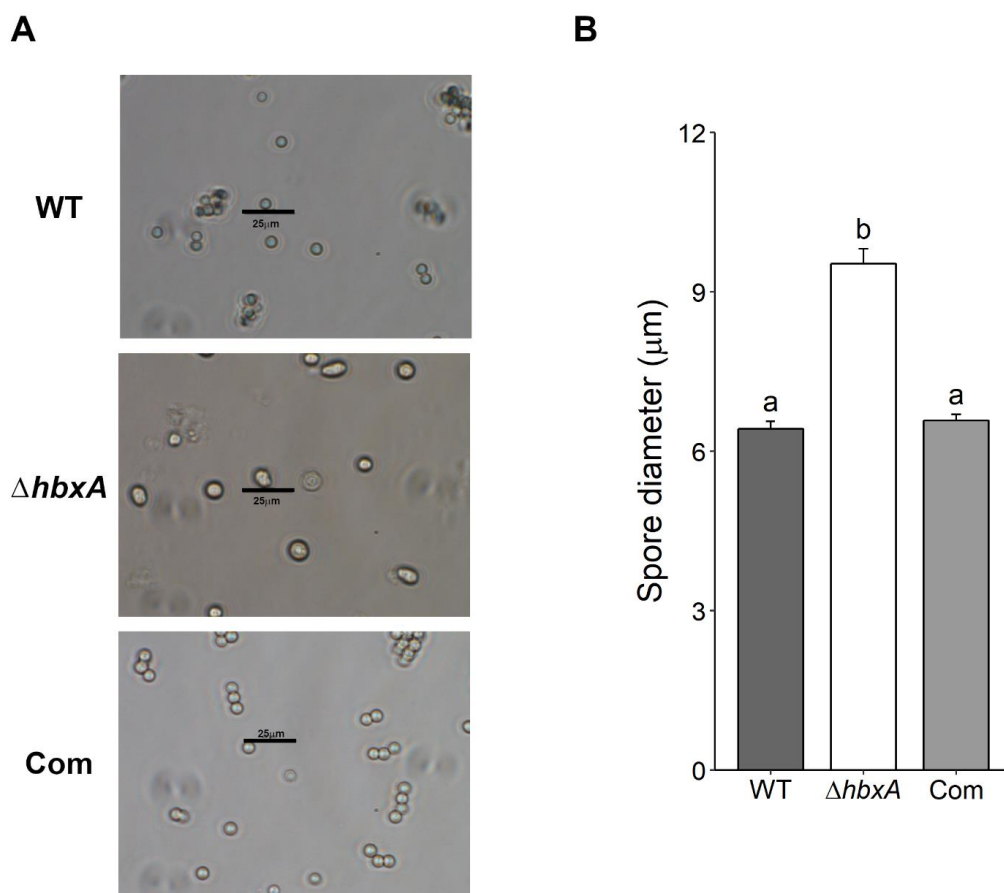
528 determined by ANOVA with the Tukey test comparison. This experiment was performed two

529 times with three biological replicates in each experiment. For qRT-PCR technical replicates were

530 also run for each biological replicate.

531

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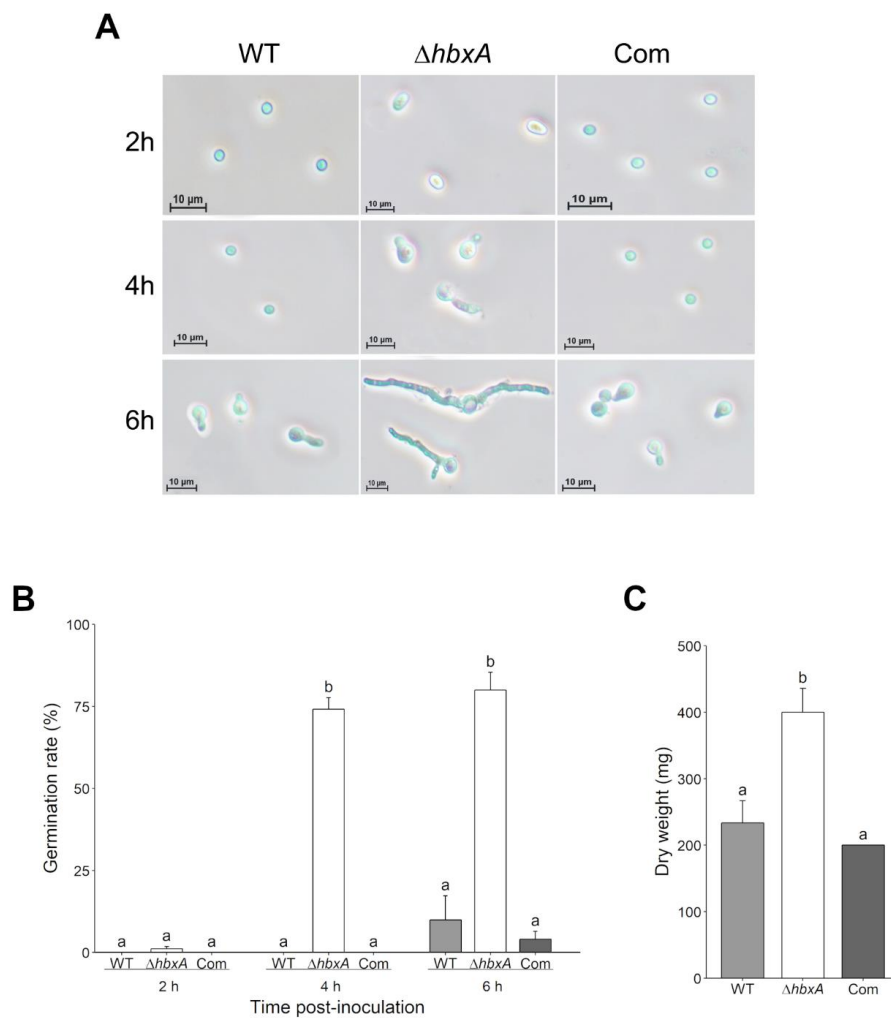


533

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

535 Wild type (WT),  $\Delta hbxA$ , 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.  
 542

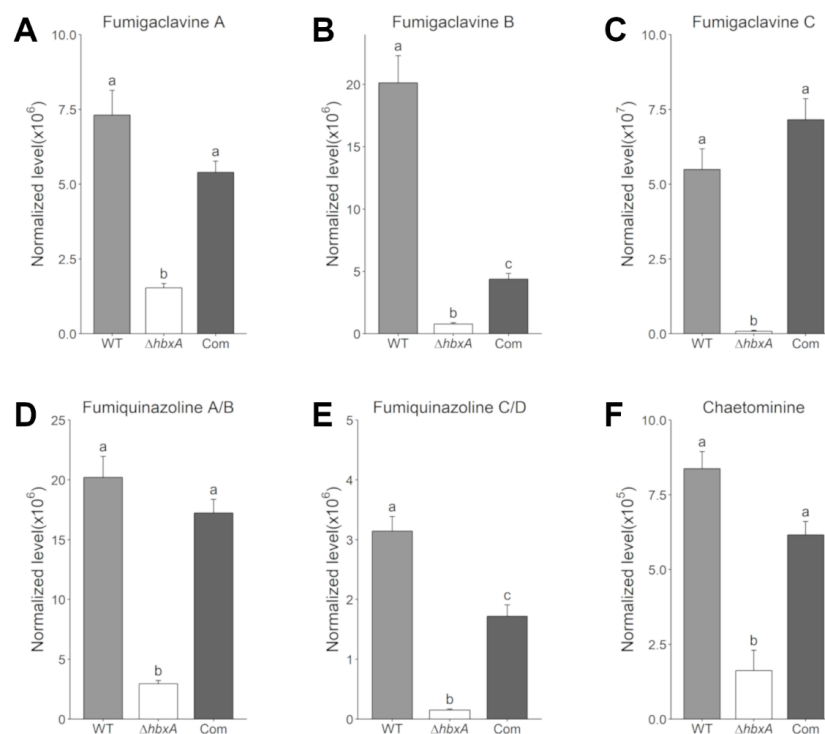


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545 **Figure 4 - Absence of *hbxA* results in earlier spore germination in *A. fumigatus***

546 Liquid GMM cultures of wild type (WT),  $\Delta hbxA$ , and complementation (Com) strains were  
 547 inoculated with  $10^6$  spores/ml. Every 2 h post-inoculation an aliquot of 500  $\mu$ l 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.



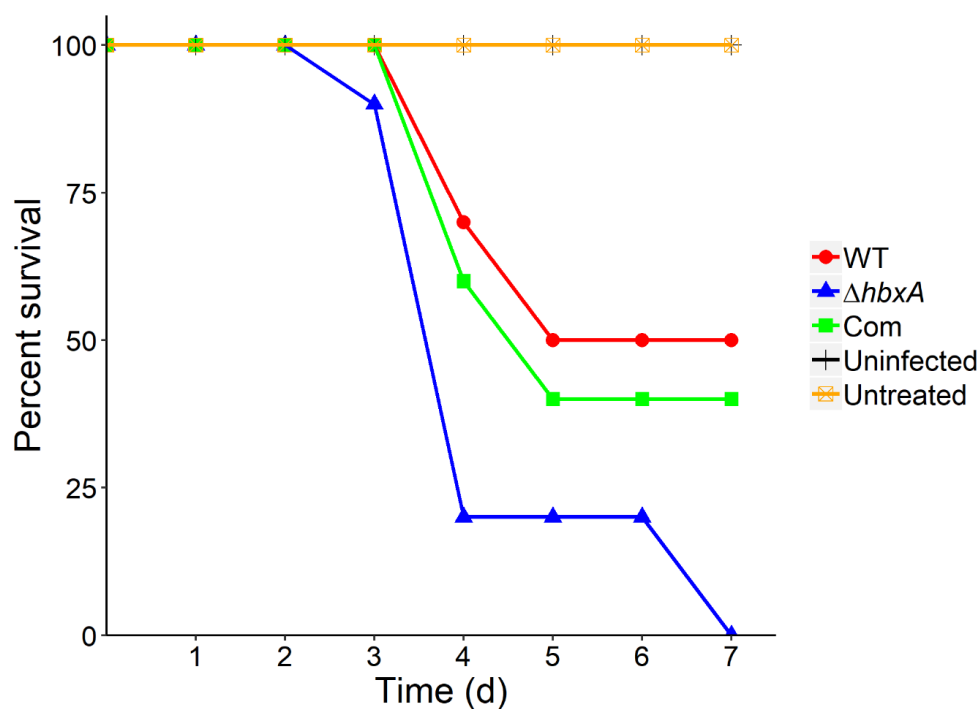
554

555 **Figure 5 – *hbxA* regulates the production of multiple secondary metabolites in *A. fumigatus***

556 Spores of *A. fumigatus* wild type (WT), deletion ( $\Delta hbxA$ ) 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.

564



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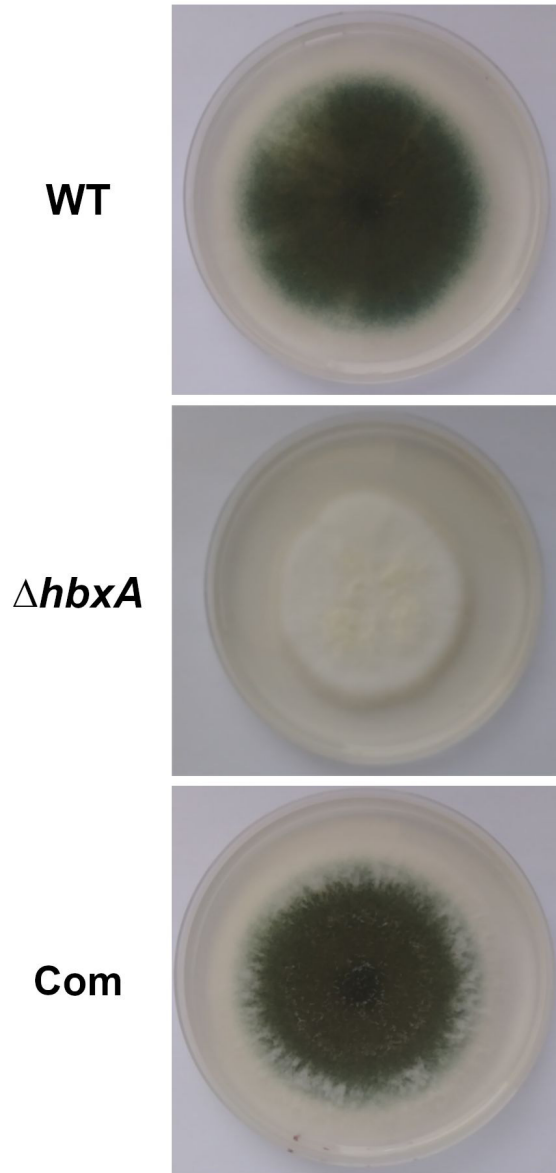
566 **Figure 6 – *hbxA* negatively regulates virulence in a mouse model**

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  $2 \times 10^6$  conidia/mouse of *A. fumigatus* wild type  
570 (WT), deletion ( $\Delta hbxA$ ) 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.



**A****B**