The hypoxia-induced dehydrogenase HorA is required for coenzyme Q10 biosynthesis, azole sensitivity and virulence of *Aspergillus fumigatus*

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Summary

*Aspergillus fumigatus* is the predominant airborne pathogenic fungus causing invasive aspergillosis in immunocompromised patients. During infection *A. fumigatus* has to adapt to oxygen-limiting conditions in inflammatory or necrotic tissue. Previously, we identified a mitochondrial protein to be highly up-regulated during hypoxic adaptation. Here, this protein was found to represent the novel oxidoreductase HorA. In *Saccharomyces cerevisiae* a homologue was shown to play a role in biosynthesis of coenzyme Q. Consistently, reduced coenzyme Q content in the generated ∆horA mutant indicated a respective function in *A. fumigatus*. Since coenzyme Q is involved in cellular respiration and maintaining cellular redox homeostasis, the strain ∆horA displayed an impaired response to both oxidative and reductive stress, a delay in germination and an accumulation of NADH. Moreover, an increased resistance against antifungal drugs was observed. All phenotypes were completely reversed by the addition of the synthetic electron carrier menadione. The deletion strain ∆horA showed significantly attenuated virulence in two murine infection models of invasive pulmonary aspergillosis. Therefore, the biosynthesis of coenzyme Q and, particularly, the fungal-specific protein HorA play a crucial role in virulence of *A. fumigatus*. Due to its absence in mammals, HorA might represent a novel therapeutic target against fungal infections.
Introduction

*Aspergillus fumigatus* is an opportunistic fungal pathogen causing invasive aspergillosis (IA) in severely immunocompromised patients (Post *et al.*, 2007). Diagnosis of IA remains difficult and treatment options for IA are still limited leading to a high mortality rate ranging from 30 to 90 % (Brakhage, 2005). The infectious agent of *A. fumigatus* are the airborne conidia, which can be easily inhaled due to their small size of 2 – 3 µm. In immunocompromised patients the inhaled spores germinate in the lung alveoli and start to grow invasively in the lung tissue (Brakhage & Langfelder, 2002). During the infection process *A. fumigatus* is challenged by massive changes of the environmental conditions, *e.g.* by nutrient depletion, elevated temperatures and hypoxia. Oxygen availability drops from 21 % (v/v) in the atmosphere to 14 % in the lung alveoli (Jain & Sznajder, 2005). In the surrounding tissue, oxygen levels are found to be between 2 to 4 % and in inflammatory and necrotic tissue even less than 1 % (Lewis *et al.*, 1999). In line, hypoxic microenvironments were found to exist at the site of infection in mice infected with *A. fumigatus* (Grahl *et al.*, 2011).

The availability of oxygen is critical for both respiration and the biosynthesis of cellular compounds such as heme, sphingolipids, sterols and the redox-active lipid coenzyme Q. In general, bacteria and eukaryotes use coenzyme Q as an electron shuttle during aerobic respiration. Coenzyme Q is a benzoquinone with an isoprenoid moiety. The number of prenyl units of the side chain depends on the species. For instance, the yeast *Saccharomyces cerevisiae* side chain consists of 6, whereas in *A. fumigatus* 10 prenyl units were found (Meganathan, 2001). However, particularly the biosynthesis of the quinone ring involves several O₂-requiring hydroxylation steps catalyzed by oxygen-dependent mono-oxygenases. Extensive studies of coenzyme Q biosynthesis in *S. cerevisiae* revealed that the oxygen level in the environment correlates well with the quantity of coenzyme Q in yeast cells (Rosenfeld
The hypoxic regulation of Q10 biosynthesis and the impact of the cellular content of coenzyme Q on adaptation to hypoxia have hardly been studied in *A. fumigatus*. Also, functional mitochondria play an essential role in the adaptation process towards hypoxia of several pathogenic fungi (Ingavale *et al.*, 2008). Though mitochondria are the main site of energy production during aerobic respiration with oxygen as terminal electron acceptor, the protein levels of all respiratory complexes also increased under hypoxic growth conditions in *A. fumigatus* most likely to increase the respiratory capacity of *A. fumigatus* mitochondria (Vodisch *et al.*, 2011). In accordance with this finding, it was shown that aerobic respiration was active during hypoxia and contributed to the virulence of *A. fumigatus* (Grahl *et al.*, 2012). All these observations indicated that mitochondria are decisive for essential fungal functions that are linked not only to energy metabolism, but also to oxidative stress, cell signaling, apoptosis and pathogenicity. Despite differences to mammals, fungal-specific mitochondrial proteins and pathways remain poorly investigated although they may provide new insights into the biology of fungi (Chatre & Ricchetti, 2014) and adaptation to hypoxic conditions.

Proteome analysis of the immediate response of *A. fumigatus* towards hypoxia revealed an increased abundance of a mitochondrial protein (Barker *et al.*, 2012). Here, it was functionally characterized as a novel hypoxia-induced oxidoreductase, designated HorA (AFUA_4G09810). HorA is associated with the biosynthesis of coenzyme Q10 and therefore mediates the activity of the electron transport chain. It is regulated by SrbA, the key transcriptional activator of hypoxic genes. Accordingly, HorA is induced under hypoxic growth conditions. However, in particular during normoxia the deletion of the *horA* gene caused reduced growth, increased resistance against antifungal drugs and an imbalance of the NADH/NAD⁺ ratio. Thus, by being important for a sufficient production of coenzyme Q, HorA plays a critical role in the virulence of *A. fumigatus*. Noteworthy, due to its absence in...
mammals the mitochondrial short chain dehydrogenase HorA may represent a promising target for the development of novel antifungal drugs.
Results

Protein HorA (AFUA_4G09810) is involved in hypoxic adaptation and conserved among fungi.

Previously, by proteome analysis of the short-term response of *A. fumigatus* towards hypoxia we found an increased abundance of the uncharacterized protein AFUA_4G09810 (Barker *et al.*, 2012). Hence, we named this protein of unknown function HorA. The hypoxia-induced expression was confirmed by Northern blot analysis showing higher transcript levels of *horA* already after 3 h of hypoxia (Fig. 1A).

A phylogenetic analysis of the HorA protein revealed that it was presumably derived from a common ancestor within the fungal kingdom (Fig. 1B). Noteworthy to mention is that the oxidoreductase HorA exhibited some similarity to the representatives of this class of proteins in lineages outside the fungal kingdom. However, this similarity was low and did not exceed the threshold of 35-40% of identity and protein length coverage that is usually used for detection of orthologs. The similarity to bacteria was 25-27%, to archaea around 30%, and to plants between 26-29%. By contrast, the similarity to fungal gene sequences was very high: the first 200 hits showed an identity of 50-99% and a coverage of 70-99%. In addition, neither Phylome DB nor MetaPhors listed non-fungal sequences. Thus, we can conclude that the oxidoreductase HorA represents a fungal-specific protein. Interestingly, representative HorA homologous proteins of the class Saccharomycetes within the phylum Ascomycota, such as *Saccharomyces cerevisiae* and *Candida glabrata*, formed an own cluster more distantly related to similar proteins in the filamentous ascomycetes. Quite recently, the orthologous protein (Coq11) of *S. cerevisiae* was described to be involved in coenzyme Q6 biosynthesis and to be part of the Q6 biosynthetic complex (Allan *et al.*, 2015).

Generation of a *ΔhorA* mutant revealed delayed germination and a growth defect on glucose-containing media.
In order to investigate the biological function of HorA in *A. fumigatus* and to determine a putative role in coenzyme Q biosynthesis, the deletion strain ΔhorA and the corresponding complemented strain horA<sup>C</sup> were generated (Fig. S1). *A. fumigatus* CEA17ΔakuB<sup>KU80</sup> (da Silva Ferreira et al., 2006) was used as a parental strain. To exclude a potential interaction of the KU80 with the horA genes we compared the transcript level of horA in CEA10 (clinical isolate) and CEA17ΔakuB<sup>KU80</sup>, which was derived from CEA10 strain. The expression of the horA gene remained the same in both strains (Fig. S8C).

To analyze the impact of HorA on the adaptation to different oxygen levels growth of *A. fumigatus* wild type, ΔhorA and horA<sup>C</sup> were studied under normoxic (21 % O<sub>2</sub>) and hypoxic (0.2% O<sub>2</sub>) conditions (Fig. 1C). Compared to the wild type and horA<sup>C</sup>, growth of ΔhorA was drastically reduced during normoxia and colonies showed a compact morphology. Surprisingly, this strong growth phenotype of ΔhorA was completely abrogated under hypoxia.

To elucidate the physiological consequences of the lack of HorA, we examined the growth phenotypes of the deletion strain under normoxic conditions. Germination of ΔhorA conidia was delayed by 12 h compared to the wild type and horA<sup>C</sup> (Fig. 2A) under normoxia. This observation was further confirmed when glucose consumption was monitored over time. Glucose consumption was delayed in ΔhorA in comparison to wild type and horA<sup>C</sup> (Fig. 2B), but growth yield remained the same for all tested strains after the consumption of 30 mM glucose.

We also tested the growth of ΔhorA on different carbon sources. ΔhorA grew slower and in a compact form when hexoses or disaccharides served as the carbon sources (Fig. S2A). When using pentoses, C2 compounds or carbon sources that are metabolized via the TCA cycle, growth of ΔhorA was still reduced, but colonies grew less compact than observed for hexoses (Fig. S2B). Interestingly, compared to the wild type and horA<sup>C</sup>, growth of ΔhorA was only
slightly reduced when peptone or casamino acids were used as sole carbon source (Fig. 2C). However, a combination of peptone and glucose as carbon sources in the medium showed that the addition of 5 mM glucose already induced compact growth of the ΔhorA deletion strain (Fig. 2C). These observations indicated that the metabolisation of glucose and hexoses was impaired in ΔhorA.

**Analysis of coenzyme Q10 content indicates involvement of HorA in coenzyme Q production.**

To determine whether the horA deletion mutant was impaired in the biosynthesis of coenzyme Q10 due to its similarity to the *S. cerevisiae* protein COQ11 we extracted mitochondria from ΔhorA and the wild type. Isolated quinone fractions from mitochondria were subjected to LC-MS/MS analysis when coenzyme Q10 was used as a standard. The analysis showed that coenzyme Q10 production in the *A. fumigatus* wild type strain was 20 times higher than in ΔhorA. Trace amounts of coenzyme Q10 were still detectable in the deletion strain (Table 1).

**Proteome analysis of ΔhorA revealed changes in the stress response.**

Coenzyme Q biosynthesis has been intensively studied in baker’s yeast, but little is known about the consequences of an impaired coenzyme Q production in filamentous fungi. For this reason, we elucidated the effect of horA gene deletion by comparing the *A. fumigatus* proteome of the wild type and the deletion strain ΔhorA. In total, 40 proteins with different abundances were identified between wild type and ΔhorA (Table S3) including proteins involved in the oxidative and nitrosative stress response and oxidative phosphorylation. Several subunits of mitochondrial respiratory complex I and the cytochrome c oxidase were up-regulated in the ΔhorA strain, while the level of proteins involved in the oxidative stress response of *A. fumigatus*, *e.g.* Cat1 and Aspf27, was strongly decreased. By contrast, the NO-detoxifying flavohemoprotein FhpA, was more abundant suggesting increased nitrosative
stress in the ΔhorA mutant. The increased level of the mitochondrial heat-shock protein HSP60 and the co-chaperone GrpE indicated some disturbance in mitochondrial protein translocation and folding in the ΔhorA strain.

**Deletion of horA affected the nitrosative and oxidative stress response.**

To confirm the results of the proteome analysis, the nitrosative and oxidative stress responses of ΔhorA were analyzed in detail. Northern blot analysis of genes encoding flavohemoprotein FhpA and S-nitrosothiol reductase GnoA (Lapp et al., 2014), both involved in NO-detoxification, was performed (Fig. 3A). In line with the proteome data, the mRNA steady-state level of fhpA was increased, whereas the gnoA mRNA level was not different in the ΔhorA mutant compared with the wild type. In addition, independent of the carbon source ΔhorA was more susceptible to DETA-NO than the wild type and the horAc strain (Fig. 3B), which implied that the mutant strain encountered increased nitrosative stress and was therefore sensitive to this exogenous NO donor molecule.

Because the levels of antioxidative proteins decreased in ΔhorA, the sensitivity of ΔhorA against reactive oxygen species (ROS) was tested. Treatment of conidia with H2O2 showed that ΔhorA spores were more susceptible to oxidative stress than the wild type and horAc (Fig. 3C). However, inhibition zone assays in the presence of the oxidative stress reagents H2O2, diamide or menadione did not reveal differences in sensitivity (Fig. S3A). Remarkably, in the presence of the electron-coupling agent menadione, which generates superoxide anions through redox cycling, no difference in the growth phenotype of *A. fumigatus* wild type, ΔhorA and horAc was observed (Fig. S3B). Even a very low concentration of 1 µM menadione restored the wild type phenotype of the ΔhorA deletion strain (Fig. 3D) and reduced transcriptional levels of catalase cat1 increased again in the presence of 1 µM menadione in ΔhorA (Fig. 3E). Besides the formation of ROS via redox cycling, menadione
can act as an electron carrier (Nosoh et al., 1968). Thus, it is likely that menadione was able to restore the electron transport system in ∆horA due to its function as electron shuttle.

To gain further insights into the connection of HorA to other cellular pathways, the corresponding gene was overexpressed in the wild type under the control of the constitutive gpdA promoter. Northern blot analysis of horA_OE confirmed the successful overexpression of horA, which was accompanied by increased expression of cat1 (Fig. S5A). In line, compared to the wild type strain horA_OE revealed an increased resistance against H₂O₂ and nitrosative stress in inhibition zone assays (Fig. S5B). Thus, the oxidative stress state of mycelia was highly affected by deleting or overexpressing horA.

**The ∆horA phenotype reflects an imbalanced intracellular redox environment.**

The decreased oxidative stress response, the reduced metabolic activity and the growth defect on glycolytic carbon sources of ∆horA, pointed to an accumulation of reduction equivalents in the cell due to depletion in coenzyme Q10. Consistently, independent of the carbon source (glucose or peptone) ∆horA exhibited an increased susceptibility against the strong reducing agent DTT (Fig. 4A). Furthermore, the quantification of the pyridine nucleotides showed a significant change of the NADH/NAD⁺ ratio due to an accumulation of NADH in the ∆horA deletion strain (Fig. 4B). There was no difference in the NADH/NAD⁺ ratio between wild type and horA_OE (data not shown). Hence, the deletion of horA led to an impaired redox homeostasis and an accumulation of NADH.

**HorA was localized in mitochondria and influenced respiratory activity and function of complex I.**

A mitochondrial targeting sequence of 27 aa was identified in the N-terminus of HorA using the prediction tool MitoProt II – v1.101. The identification of the signal peptide by MALDI-TOF/TOF analysis of the tryptically digested HorA spot (see proteome analysis) indicated...
that it contains an uncleaved signal for mitochondrial import (Fig. S6). Due to its involvement in the biosynthesis of coenzyme Q10 and the presence of a mitochondrial target sequence, HorA is most likely located at the matrix side of the inner mitochondrial membrane (Gonzalez-Mariscal et al., 2014).

To analyze the mitochondrial localization of HorA, eGFP was fused to the C-terminus of HorA. The expression of the gene construct was controlled by the *horA* native promoter. The construct was ectopically integrated into the genome of *A. fumigatus* wild type (Fig. S7). The green fluorescent signal of the *horA* eGFP fusion strain was found in the mitochondrial network of hyphae (Fig. 4C). This observation was further confirmed by an overlap of the eGFP signal with the red fluorescence signal of the mitochondria-specific dye Mitotracker Deep Red (Fig. 4C). In addition, measuring the enzymatic activities in isolated mitochondria from the wild type and ∆*horA* provided evidence of an impaired activity of complex I (Table 2). In the mutant, the activity of NADH:ubiquinone oxidoreductase (complex I) was 39.8 nmol min⁻¹ mg⁻¹, and therefore reduced to 20% compared with the activity measured in the wild type (226.0 nmol min⁻¹ mg⁻¹). Interestingly, the activity of the alternative NADH:ubiquinone oxidoreductase (rotenone insensitive) was higher in the ∆*horA* deletion strain than in the wild type, which might indicate a compensatory mechanism. Further measurements revealed that there was only a slight difference in complex III activity between the tested strains, whereas activity of cytochrome C oxidase (complex IV) was two times higher in strain ∆*horA* in contrast to the wild type. Therefore, significant reduction of complex I activity in the *horA* deletion strain suggests a significant contribution of the putative coenzyme Q biosynthesis enzyme HorA to electron translocation by complex I. When the strain ∆*horA* was cultivated in AMM in the presence of menadione, the enzymatic activity of complex I was insignificantly changed in comparison to the wild type strain (data not shown).
The lack of horA induced antifungal drug resistance.

It was shown that dysfunctional mitochondria affect lipid homeostasis and thus cause an activation of the multidrug resistance pathway in fungi (Shingu-Vazquez & Traven, 2011). To test, whether ΔhorA was more resistant against antifungal drugs, A. fumigatus wild type, ΔhorA and horA<sup>C</sup> were cultivated in the presence of amphotericin B, voriconazole and terbinafine (Fig. 5). In contrast to the wild type and horA<sup>C</sup>, ΔhorA was able to grow in the presence of higher concentrations of voriconazole (0.25 µg mL<sup>-1</sup>) and amphotericin B (5 µg mL<sup>-1</sup>). Although terbinafine had no effect on ΔhorA, it reduced growth of the wild type and horA<sup>C</sup>. In addition, we examined the drug susceptibility of the mutant when peptone was used as a carbon source. The partially abolished growth defect in the presence of peptone did not change drug sensitivity of the mutant. The mutant strain still showed a higher tolerance to antifungals than the wild type (Fig S4). MIC values of tested antifungal compounds were identified by the resazurin microtiter assay with AMM as the growth medium (Monteiro et al., 2012). The identified voriconazole and terbinafine MIC values of the ΔhorA and the wild type strain confirmed the higher antifungal resistance of the ΔhorA mutant (Table S4). However, the amphotericin B MIC value of ΔhorA did not differ from the wild type. Most likely ΔhorA was able to develop resistance against amphotericin B only during growth on solid media, on which the growth rate was lower and some mycelia were exposed to an air interphase.

Besides impaired mitochondria, also the overexpression of ergosterol biosynthesis genes or changes in the permeability of the cytoplasmic membrane can cause resistance against azoles and polyenes in fungi (Masia Canuto & Gutierrez Rodero, 2002). However, Northern blot analysis of the expression of several genes involved in ergosterol biosynthesis such as hmgA, erg10, erg11, erg13, erg24, erg25 revealed no difference between wild type and ΔhorA (data not shown). Taken together, these findings suggest that the increased resistance of ΔhorA
against antifungal drugs was caused by impaired function of mitochondria, which might be accompanied by the induction of the multidrug resistance pathway.

**horA is regulated by the hypoxia and triazole drug response regulator SrbA.**

Since HorA was shown to be more abundant during the short-term response of *A. fumigatus* towards hypoxia, we investigated whether *horA* is a target gene of the transcriptional regulator SrbA. As aforementioned, this SREBP transcriptional regulator is known to mediate cellular responses against azoles and hypoxia in *A. fumigatus* (Blatzer *et al.*, 2011). By analyzing microarray data published by Blatzer *et al.* (2011) we found the transcript level of the *horA* gene to be downregulated in the ΔsrbA mutant after shifting the culture from normoxia to hypoxia by a factor of 3.7, 3.4 and 3.9 after one, two and four hours of hypoxia induction, respectively. To confirm these results we cultivated the *A. fumigatus* srbA knockout strain for 16 hours in a shaking flask and subsequently shifted the culture for six hours to hypoxic conditions (0.2% O₂). Transcript levels of *horA* were determined before and after the hypoxic shift by Northern blot. A lack of SrbA led to a significant downregulation of *horA* mRNA levels under hypoxic conditions, which suggests that SrbA either directly or indirectly activates the *horA* gene transcription at low O₂ levels (Fig S8B). To further confirm that *horA* represents a target gene of SrbA, the promoter region of *horA* was searched for binding sites of SrbA. Indeed, an SrbA binding site motif (ATCTCCTCATC) (Chung *et al.*, 2014) was found 25 bp upstream of the transcription start site (Fig. S8A).

**Strain ΔhorA is almost avirulent in a chicken embryo and two independent murine infection models.**

To show whether HorA is essential for *A. fumigatus* to establish an infection, wild type and Δ*horA* were tested in a chicken embryo infection model (Fig. S9A). Compared to the wild type, Δ*horA* was significantly reduced in virulence. Additionally, the transcription of *horA*...
was analyzed in lungs of infected mice (Fig. S9B). Expression of the citrate synthase gene \textit{cit1} was monitored as positive control for \textit{A. fumigatus} infection. Both \textit{cit1} and \textit{horA} were expressed during infection. For this reason, \textit{A. fumigatus} wild type, \textit{ΔhorA} and \textit{horA}\textsuperscript{C} were tested for virulence in two different murine infection models of IA. In the leukopenic model, mice were immunosuppressed with cyclophosphamide and a single dose of cortisone acetate. Using solely cortisone acetate for immune suppression, the killing activity of phagocytes is reduced but neutrophils are still recruited to the site of infection. Remarkably, the deletion of \textit{horA} resulted in the attenuation of virulence in both infection models (Fig. 6A, 6C). In the cortisone acetate model 90 % of the mice infected with the \textit{ΔhorA} strain survived, whereas the survival was only 10 % for mice infected with conidia of the wild type or \textit{horA}\textsuperscript{C} strain (Fig. 6A). Similarly, in the leukopenic model 90 % of mice infected with the \textit{ΔhorA} strain survived. By contrast, only 20 % of mice survived when infected with the wild type and no mouse survived an infection with the \textit{horA}\textsuperscript{C} strain (Fig. 6C). In most cases, mouse lungs infected with the \textit{ΔhorA} mutant strain and sacrificed at the end of the infection experiment (14 days) did not show any signs of fungal growth or tissue destruction (Fig. 6B, 6D). By contrast, sections of lungs infected with wild type or \textit{horA}\textsuperscript{C} showed fungal growth and the recruitment of immune cells. However, in some sections of lungs from mice infected with the \textit{ΔhorA}, fungal growth was observed after 11 and 14 days in the cortisone acetate infection model and after 14 days in the cyclophosphamide infection model (Fig. 6B, 6D). This suggests that in principle the \textit{ΔhorA} strain was able to grow in the lungs, but mostly it was cleared by the murine immune system and no colonization in the lungs could be established.
**Discussion**

Being linked to energy metabolism, oxidative stress and cell signaling, fungal mitochondria are required for many essential functions including pathogenicity and drug tolerance. Here, we describe the impact of mitochondrial dysfunction caused by lack of the fungus-specific coenzyme Q biosynthesis oxidoreductase HorA on growth, drug resistance and virulence of *A. fumigatus*. It must be acknowledged that our study on the *horA* gene function was performed in an *A. fumigatus* wild type strain, which carries a deletion of the *akuB*^KU80^ gene. The KU80 protein is involved in repairing DNA double strand breaks and maintaining the telomeric structure (Featherstone & Jackson, 1999). It cannot be absolutely excluded that an interaction occurs between HorA and KU80. However, we assume that an interaction between a coenzyme Q biosynthetic protein and a DNA repair protein is relatively unlikely and the observed phenotypes in this study are not caused by the lack of KU80.

HorA was previously identified in the proteome map of resting conidia and found to be up-regulated within the first 24 h of adaptation to hypoxia and putatively regulated by the transcriptional activator SrbA (Barker *et al.*, 2012, Teutschbein *et al.*, 2010, Hillmann *et al.*, 2014). Here, we confirmed the SrbA-dependent regulation and found a putative SrbA-binding site in the promoter region of the *horA* gene. In line, genes required for the oxygen-dependent coenzyme Q biosynthesis were also upregulated under anaerobic conditions in the fungus *Schizosaccharomyces pombe* and required Sre1p, the SrbA ortholog in this yeast (Todd *et al.*, 2006). Similarly, several coenzyme Q biosynthesis genes (*coq*) were up-regulated in *A. fumigatus* after a short period of hypoxia (Hillmann *et al.*, 2014). The observed upregulation of *coq* genes is consistent with the hypothesis that oxygen-requiring pathways are upregulated during hypoxia to maintain flux through these pathways when oxygen levels are low (Todd *et al.*, 2006).
During this work a homologous protein to HorA was described in *S. cerevisiae* and designated as Coq11 (Allan *et al.*, 2015). The authors found this polypeptide to be associated with the coenzyme Q biosynthetic complex (the CoQ-synthome), particularly with Coq4, Coq5, and Coq7. However, the biological function of Coq11 remained unclear. It was suggested that Coq11 catalyzes an FMN-dependent decarboxylation step in the coenzyme Q biosynthesis process. Taking into consideration the putative involvement of Coq11 in Q biosynthesis and the upregulation of *A. fumigatus* HorA during hypoxia, it is conceivable that the HorA-dependent step of coenzyme Q biosynthesis is differentially regulated and important for respiration of *A. fumigatus* under hypoxia. Furthermore, the absence of the growth defect of the ∆horA mutant under low oxygen environments in contrast to normoxic conditions suggests that for electron shuttling *A. fumigatus* uses coenzyme Q intermediates or alternative mechanisms when O$_2$ levels drop and production of the O$_2$-dependent coenzyme Q10 biosynthesis is impaired. Alternatively, the reduced metabolic flux and decreased production of NADH under hypoxia may lead to a less drastic phenotype.

The predicted mitochondrial localization was confirmed by specific accumulation of the HorA_eGFP fusion protein in mitochondria. The mitochondrial target sequence of HorA contains an NAD(P)H cofactor binding site. Remarkably, by mass spectrometric analysis tryptic peptides derived from the signal peptide of HorA were identified which suggests that no cleavage of the precursor signal occurs. This phenomenon has been described before for mitochondrial outer membrane proteins (Shore *et al.*, 1995) and a few specific matrix proteins (Waltner & Weiner, 1995). Since the amino acid sequence of HorA does not contain a transmembrane domain, a direct integration of HorA into the mitochondrial membrane appears very unlikely. Instead, an association with the coenzyme Q biosynthesis complex can be assumed, which is peripherally located at the matrix side of the inner mitochondrial membrane (Tran & Clarke, 2007).
Ubiquinone plays an essential role in energy production and redox homeostasis. During metabolism of sugars, both the glycolysis and the TCA cycle cause a high rate of reduction of NAD\(^+\) to NADH. Interestingly, growth of \(\Delta horA\) was strongly inhibited in the presence of glycolytic carbon sources indicating an imbalance of the NADH/NAD\(^+\) ratio in the absence of a proper amount of coenzyme Q10. When glucose was employed as carbon source, in the \(\Delta horA\) mutant strain NADH accumulated and the NADH/NAD\(^+\) ratio significantly increased. This resulted in the impaired complex I-mediated regeneration of NAD\(^+\) in \(\Delta horA\).

Complex I and III are the main sites for mitochondrial ROS production. In contrast to complex III, in complex I the specific site of superoxide anion production has not been identified yet (Grivennikova & Vinogradov, 2006, Jezek & Hlavata, 2005). It was proposed, that a high turnover rate of NADH to NAD\(^+\) by complex I leads to an increased production of superoxide anions which can be further converted to the highly reactive hydrogen peroxide by superoxide dismutase (Murphy, 2009). The comparison of the proteome of \(A. fumigatus\) wild type and the deletion strain \(\Delta horA\) revealed a down-regulation of the oxidative stress response in the deletion strain. Both, the lower abundance of catalase Cat1 and the increased susceptibility of \(\Delta horA\) conidia against hydrogen peroxide indicate less ROS production in the knock-out strain \(\Delta horA\) due to decreased metabolic activity of complex I. In contrast to the oxidative stress response, the NO detoxifying flavohemoprotein FhpA was highly up-regulated in \(\Delta horA\). Furthermore, growth of \(\Delta horA\) was inhibited in the presence of the NO-donor DETA-NO indicating an activation of the nitrosative stress response (de Jesus-Berrios et al., 2003, Lapp et al., 2014).

Taken together, our results provide further evidence that the deletion of \(horA\) impairs the biosynthesis of ubiquinone and as a consequence, leads to a reduced activity of complex I. This causes an accumulation of NADH and a reduced production of superoxide anions resulting in an imbalanced redox state of the deletion strain. This hypothesis was further
supported by the increased sensitivity of $\Delta_{horA}$ against DTT. The reducing agent DTT affects the intracellular redox homeostasis by reduction of disulfide bonds and thereby activates the unfolded protein response (Richie et al., 2009). Hence, the deletion of $horA$ caused an imbalance of the redox state leading to increased reductive stress.

In the $\Delta_{horA}$ mutant strain all phenotypes were reversed by the addition of menadione, which can potentially serve as an electron shuttle. It is very likely that menadione was used as a substrate for reduction by NAD(P)H:quinone acceptor oxidoreductase (NQO) in the $\Delta_{horA}$ mutant of $A. fumigatus$. The NQO enzyme can mediate the reduction of short-chain quinones and bypass mitochondrial complex I (Dinkova-Kostova & Talalay, 2010, Haefeli et al., 2011). In line, in the presence of menadione the $horA$ deletion strain did not exhibit a fully restored enzymatic activity of complex I. In contrast, measured activities of complex III and IV in the $\Delta_{horA}$ strain cultivated with menadione were not different from activities of these complexes measured in wild type (data not shown). Therefore, in the $\Delta_{horA}$ strain the NQO enzyme may reduce menadione that is coupled to NADH oxidation, which helps the fungus to avoid redox imbalance caused by NADH accumulation. Interestingly, to rescue complex I deficient cells quinones have to meet certain requirements. For instance, the polarity should allow a quinone to be reduced by cytosolic NQO enzymes and at the same time, the quinone must be able to enter mitochondria to release electrons to complex III (Haefeli et al., 2011). Apparently, only menadione is able to carry out so called cytosolic-mitochondrial electron shuttling in $A. fumigatus$. Accordingly, in yeast except of menadione no other quinone was able to bridge the electron transfer of NADH oxidation when complex III was inhibited (Brivet-Chevillotte & di Rago, 1989, Nosoh et al., 1968). Likewise, menadione restored the electron flow and ATP formation in cardiomyocytes after the inhibition of complex I with rotenone (Shneyvays et al., 2005).
Another interesting finding was the antifungal drug resistance of ∆horA. In yeast, dysfunctional mitochondria affected lipid homeostasis and thus caused an activation of the multidrug resistance (MDR) pathway (Shingu-Vazquez & Traven, 2011). Azole-resistant clinical isolates of Candida albicans showed an impaired function of mitochondria and changes in membrane composition of plasma domains and mitochondria (Singh et al., 2012). In the yeast C. glabrata, the activation of the alternative oxidase pathway induced resistance against azoles (Peng et al., 2012). Furthermore, azole-resistant C. glabrata isolates displayed a reduced ROS production (Peng et al., 2012). Several other reports also described the connection between mitochondria dysfunction and drug resistance in fungi. For instance, a recent study of mitochondria dynamics showed that mitochondrial fission mutants of A. fumigatus exhibited increased azole resistance that was suggested to be linked to lanosterol 14α-demethylation activity (Neubauer et al., 2015). Similarly, the A. fumigatus ∆horA mutant strain showed an increased resistance against voriconazole and amphotericin B. However, expression of target genes of these antifungals in the ergosterol biosynthesis was not affected in the deletion strain. Recently, a screen of A. fumigatus mutants identified triose phosphate isomerase and the 29.9 kDa subunit of complex I as mediators for azole sensitivity (Bowyer et al., 2012). In Neurospora crassa, the deletion of the 29.9 kDa subunit affected the transition between activation and deactivation of complex I (Ushakova et al., 2005). Triose phosphate isomerase and the 29.9 kDa subunit of complex I are involved in NAD\(^+\) regeneration and thus the authors hypothesized that mitochondrial NADH metabolism contributes to azole drug response in A. fumigatus (Bowyer et al., 2012). It is thus most likely that the decreased susceptibility of A. fumigatus ∆horA against antifungal drugs was caused by the impaired function of mitochondria and the dramatic alteration of the NADH/NAD\(^+\) ratio. Thus, this metabolic state confers resistance against antifungal drugs.
An additional major outcome of this study is the attenuated virulence of the *A. fumigatus* ΔhorA mutant strain in both mouse infection models applied. Histopathological analysis of the infected mice lungs proved that the inhaled conidia of ΔhorA were unable to colonize the lung. This result is in agreement with the observed reduced fungal burden and impaired virulence of *A. fumigatus* ΔcycA (Grahl et al., 2012). Both *A. fumigatus* ΔhorA and ΔcycA mutants exhibited a delay in germination which may contribute to the attenuated virulence. However, a delay in germination does not always lead to the inability to cause disease, as shown for an *A. fumigatus* mutant deleted in the HOG-MPKA pathway sensor Sho1 (Ma et al., 2008). The germination time of ΔhorA depended on the carbon source available. With glucose, germination of *A. fumigatus* ΔhorA was significantly delayed. By contrast, when peptone was the only carbon source the growth defect of ΔhorA was abrogated. Thus, it is interesting to speculate, that at the onset of infection in mice lungs, hexoses or other readily metabolizable carbon sources are present. However, compared to the *A. fumigatus* wild type, the mortality rate caused by the ΔhorA mutant was reduced in an egg infection model, where proteins are the main carbon source. Hence, we hypothesize that the delay in germination of *A. fumigatus* ΔhorA is not the main factor contributing to the attenuation of virulence. It is thus an exciting finding that this fungus-specific protein of coenzyme Q biosynthesis is essential for virulence. Since no orthologs of HorA exist in higher eukaryotes, HorA represents an interesting target for the development of non azole-based antifungal compounds. In fact, a number of fungicides target the mitochondrial activity of phytopathogenic fungi (Fernandez-Ortuno et al., 2008).
Experimental Procedures

Ethics Statement.

All animals were cared for in strict accordance with the European animal welfare regulation. The protocol was approved by the responsible Federal State authority in Thuringia (Thüringer Landesamt für Lebensmittelsicherheit und Verbraucherschutz) and ethics committee (beratende Kommission nach §15 Abs. 1 Tierschutzgesetz) in accordance with the German animal welfare act (permit no. 03-004/12).

Strain and culture conditions.

Deletion of horA.

*A. fumigatus* was cultivated in *Aspergillus* minimal medium (AMM) containing 60 mM glucose and 70 mM NaNO\textsubscript{3} as sole carbon and nitrogen source, respectively (Brakhage & Van den Brulle, 1995). Liquid cultures were inoculated with 1×10\textsuperscript{5} conidia mL\textsuperscript{-1} AMM and incubated on a rotary shaker with 200 rpm at 37 °C for indicated time periods. Growth tests were performed on AMM 2 % (w/v) agar plates containing either 60 mM glucose or 1 % (w/v) peptone as sole carbon source. All strains used in this study are listed in Supplementary Table S1.

Generation of mutants.

*A. fumigatus* CEA17\textdelta akuB\textsuperscript{KU80} (da Silva Ferreira et al., 2006) was used as a parental strain, here defined as wild type, to generate the horA deletion strain by homologous recombination following the transformation of protoplasts (Weidner et al., 1998). For deletion of horA (AFUA_4G09810) the flanking regions were amplified from genomic DNA with the primer pairs 4G09810\_LF\_fw/4G0810\_LF\_hph\_rev and 4G09810\_RF\_hph\_fw/4G09810\_RF\_rev. By this reaction, overlapping ends to the hygromycin resistance cassette were introduced at the 3'-end of the upstream flanking region and at the 5'-end of the downstream flanking.
region of the horA gene. The hygromycin resistance cassette was amplified from plasmid pUCHph (Liebmann et al., 2004) using primers Hph_fw and Hph_rev. The horA deletion construct was obtained by a 3-fragment PCR using primers 4G09810_LF_fw and 4G09810_RF_rev.

**Complementation of the ΔhorA strain.**

To complement the ΔhorA deletion strain, 1 kbp of the native promoter, the horA gene and 300 bp of the native terminator were amplified from genomic DNA of *A. fumigatus CEA17ΔakuB* Ku80 using primers Pro_4G09810_NotI_rev and 4G09810_Term_NotI_fw. The obtained DNA fragment was inserted into the vector pSK275 (Szewczyk & Krappmann, 2010) in front of the pyrithiamine resistance cassette. The resulting vector pSK275_horA_C_PtrA was ectopically integrated into the genome of *A. fumigatus ΔhorA* by transformation of protoplasts.

**C-terminal eGFP fusion of HorA.**

To generate a C-terminal eGFP fusion protein of HorA, 1 kbp of the native promoter and the horA gene were amplified from genomic DNA of *A. fumigatus CEA17ΔakuB* Ku80 using the primers Pro_4G09810_SbfI_rev and 4G09810_XmaI-fw. The obtained DNA fragment was inserted into the vector p123_eGFP (Aichinger et al., 2003) containing the hygromycin resistance cassette. The resulting vector p123_horA_eGFP_hph was ectopically integrated into the genome of *A. fumigatus CEA17ΔakuB* Ku80.

**Generation of the horA overexpression strain.**

To constitutively express the horA gene, 1 kbp of the gpdA promoter was amplified from genomic DNA of *A. fumigatus CEA17ΔakuB* Ku80 using primers Pro_gpdA_DraIII_fwd and Pro_gpdA_BamHI_rev. Additionally, the horA gene and 300 bp of the native terminator were amplified with primers 4G09810_Term_NotI_fwd and 4G09810_BamHI_rev. The obtained DNA fragments were digested with the respective restriction enzymes according to the
indicated restriction sites of the primers. Both DNA fragments were ligated via Dral III and NotI restriction sites into the vector pSK275 (Szweczyk & Krappmann, 2010) in front of the pyrithiamine resistance cassette. The resulting vector pSK275_horAOE_PtrA was ectopically integrated into the genome of *A. fumigatus* CEA17ΔakuB*KU80*.

Transformants were selected on AMM agar plates containing either 0.1 µg/ml pyrithiamine (Sigma-Aldrich, Germany) or 240 µg/ml hygromycin (Roche Applied Science, Germany). All PCR reactions were performed with Phusion High-Fidelity DNA Polymerase (Thermo Fisher Scientific, Germany) according to the manufacturer's recommendations. All oligonucleotides used for the generation of mutants are listed in Supplementary Table S2.

**Southern blot, RNA extraction and gene expression analysis.**

*A. fumigatus* mutant strains were verified by Southern blotting. Briefly, genomic DNA of *A. fumigatus* was extracted using the MasterPure yeast DNA purification kit (Epicentre Biotechnologies, USA) and digested by specific restriction enzymes (New England Biolabs, Germany). Resulting DNA fragments were separated by agarose gel (1% w/v) electrophoresis and transferred onto Hybond™-N+ membranes (GE Healthcare Bio-Sciences, Germany) by capillary blotting. Gene specific DNA probes were generated by PCR including digoxigenin labeled dUTPs (Jena Bioscience, Germany). DIG-labeled probes were hybridized with DIG Easy Hyb and detected using Anti-digoxigenin antibody and CDP-Star ready-to-use kit (Roche Applied Science, Germany) according to the manufacturer’s protocol (Roche). Chemiluminescence signals were detected with an x-ray film.

For Northern blot analysis the mycelium was disrupted by glass beads and the RNA was extracted by TRIsure™ (Bioline, Germany) according to the manufacturer’s protocol. 10 µg of total RNA were separated on a denaturing agarose gel (1.2% (w/v) agarose, 40 mM MOPS, 10 mM sodium acetate, 2 mM EDTA, 2% (v/v) formaldehyde, pH 7). Blotting,
hybridization and detection with DIG-labeled DNA probes were performed as described above for Southern blots.

RNA of ground mice lungs was extracted with the Qiagen “RNeasy Mini Kit” (Qiagen, Germany) according to the manufacturer’s instructions. For the preparation of first-strand cDNA, RNA samples were incubated with Turbo DNA-freeTM Kit (Ambion, USA) and transcribed in cDNA with the RevertAidTM Premium First Strand cDNA Synthesis Kit (Fermentas, Germany) according to the manufacturer's protocol.

**Extraction of coenzyme Q10.**

Extraction of coenzyme Q10 from lyophilized mitochondria was implemented according to (Miyadera et al., 2001). The extract was resuspended in 500 µl of ethanol and 100 µl was injected onto an LC-MS system consisting of an HPLC: UltiMate 3000 binary RSLC with photo diode array detector (Thermo Fisher Scientific, Dreieich, Germany) and the mass spectrometer (LTQ XL Linear Ion Trap from Thermo Fisher Scientific, Dreieich, Germany) with an electrospray ion source. For HPLC analysis, an ACCUCORE RP-MS 2.6 µm 150x4.6 mm column (Thermo Fisher Scientific, Dreieich, Germany) was applied. The mobile phase consisted of an isocratic gradient of acetonitrile and isopropanol (8:7, v/v) containing 0.5% (v/v) formic acid (Zu et al., 2006). The run was 5 minutes in length and quantification was completed using the mass spectrometer in positive mode. A standard curve was obtained using coenzyme Q10 authentic standard (Sigma Aldrich, Taufkirchen, Germany) with the following concentrations: 0.115, 0.23 0.47 0.94 1.8 75 3.75 µg/ml. The Xcalibur Quan Browser software (Thermo Fisher Scientific, Dreieich, Germany) was used to calculate the amount of coenzyme Q in mitochondria of the wild type and ΔhorA. Measurements were performed on two independent biological replicates.

**Germination, growth and inhibition zone assays.**
5 mL of AMM were inoculated with $1 \times 10^6$ conidia. 50 µL aliquots of the spore suspensions were cultivated on coverslips and incubated at 37 °C in a wet chamber. Starting after 3 h of incubation, the number of germinated and non-germinated conidia was determined over time by light microscopy (Leica DM 4500B, Leica Microsystems, Germany) until all spores of the different strains were fully germinated. The germination rate was calculated as percentage of germlings in a group of at least 100 conidia. Experiments were carried out in three biological replicates.

Growth assays for the characterization of the deletion strain were performed as described earlier (Valiante et al., 2008). Briefly, freshly harvested conidia were diluted serially in sterile water in order to obtain defined spore concentrations. $10^5$, $10^4$, $10^3$ and $10^2$ conidia were spotted in a volume of 5 µl on AMM agar plates in the presence of specific agents as indicated. Normoxic or hypoxic atmospheres composed of 0.2 % O$_2$ and 5 % CO$_2$ (H35 Hypoxystation, Don Whitley Scientific, UK) were used to monitor growth of A. fumigatus on AMM agar plates. Growth was documented after two to four days of incubation at 37 °C. Experiments were performed in three biological replicates.

To determine MIC values of antifungal drugs the method based on resazurin was applied (Monteiro et al., 2012). AMM was used as the medium. Strains were cultivated in the presence of antifungals for 48h. The assessment of inhibitory concentrations was performed in three biological replicates.

Petri dishes with 10 mL AMM bottom-agar were overlaid with 10 mL AMM top-agar containing $1 \times 10^8$ conidia. In the middle of the agar plate a hole of 10 mm diameter was punched. The well was filled with 150 µL of 45 µM DETA-NO (Sigma-Aldrich, Germany), 100 mM diamide solution (Sigma-Aldrich, Germany), 1 mM menadione (Sigma-Aldrich, Germany) or 3 % (v/v) H$_2$O$_2$ (Fluka, Germany), respectively. The diameter of the inhibition
zone was measured after 16 h incubation of the agar plates at 37 °C. Experiments were performed in three biological replicates.

**Conidia sensitivity assays against oxidative stress.**

To analyze the susceptibility of conidia against oxidative stress, 1×10⁵ spores were treated with 0.2 M H₂O₂ in a total volume of 1 mL for 30 min at room temperature. After treatment spore solutions were diluted in water containing 0.001 % (v/v) Tween 80 to a concentration of 1 x 10³ conidia mL⁻¹. 100 µL of each sample were plated on an AMM agar plate containing 1 % (w/v) peptone as carbon source in two technical replicates. After 24 h incubation at 37 °C colony forming units (CFU) were counted and the relative survival was calculated in reference to the untreated control. Three independent biological replicates were analyzed.

**Determination of cell dry weight and glucose concentration.**

Cultures of 100 mL AMM were inoculated with 1×10⁷ conidia of the respective strains and incubated at 37 °C and 200 rpm. The mycelia were harvested using Miracloth (Calbiochem, Germany) when half of the added glucose was consumed. The mycelia were thoroughly dried for 5 days at 60 °C. Afterwards mycelial dry weight was determined by using an analytical balance (Kern, Germany). Experiments were done in three biological replicates.

Glucose concentration of the culture supernatant was measured over time with the BIOSEN C-Line analyzer (EKF Diagnostic, Germany) according to the manufacturer’s instructions.

**Determination of the intracellular NADH/NAD⁺ ratio.**

For the extraction of pyridine nucleotides 100 mg of ground mycelium was resuspended in 1 mL PBS buffer pH 7.5, incubated on ice for 15 min and centrifuged at 4 °C for 10 min at 15,600 × g. To quantify the concentration of intracellular NAD⁺ and NADH the Amplite™ Colorimetric NADH/NAD⁺ assay kit (AAT Bioquest, USA) was used according to the
manufacturer’s instructions. Increase of absorbance was measured over 60 min at 575 nm in a microtiter plate reader (Infinite 200 Pro, Tecan, Switzerland). Intracellular NAD\(^+\) and NADH concentrations were calculated in reference to the extracted protein concentration of each sample. Data were analyzed from three biological replicates.

**Microscopy studies.**

2×10\(^4\) conidia per ml of AMM were seeded on coverslips in a 24 well plate (Thermo Scientific Nunclon, Germany) and incubated for 20 h at 37 °C. Due to the germination defect of ΔhorA, the strain was pre-cultivated for additional 12 h at 37 °C. Cultures were stained with the mitochondrial fluorescence stain Mitotracker Deep Red (Life technologies, Germany) with a final concentration of 500 nM. After a further incubation of 1 h at 37 °C samples were washed three times with AMM. Stained and washed fungal samples on coverslips were transferred to glass slides and subjected to confocal laser scanning microscopy (CLSM). Samples were visualized using a Zeiss LSM 710 CLSM (Carl Zeiss, Germany) and analyzed with the Zeiss ZEN 2011 software (Carl Zeiss, Germany) in spectral scan mode. Unstained fungal samples were used for autofluorescence reference spectra and single stained samples for reference spectra of GFP and MitoTracker Deep Red. Microscopic pictures of the bi-fluorescent samples were generated in online fingerprinting mode using the respective reference spectra excited with an Argon-Multiline laser (488 nm) and a HeNe-laser (543nm).

**Purification of mitochondria and determination of respiratory activities.**

To purify mitochondria from *A. fumigatus* strains, a modified method described by Lang *et al.* (Lang *et al.*, 2011) was applied. Mycelia (approximately 12 g moist weight) were ground in liquid nitrogen with mortar and pestle. Obtained biomass was resuspended in 100 ml of freshly prepared cooled buffer for cell disruption (0.3 M sorbitol, 3 mM EDTA, 25 mM
MOPS, pH 7.8, 2 mM DTT, 0.1% (w/v) BSA, 0.2 mM PMSF). The crude extract was passed through two layers of Miracloth (Calbiochem). The filtrate was poured into tubes and centrifuged at 1,500 ×g for 10 min. The supernatant was transferred into new tubes and centrifuged at 3,000 × g for 5 min followed by 6,000 × g for 5 min. The supernatant containing mitochondria was transferred into new tubes and spun down at 18,000 × g for 20 min. Pellets were resuspended in 2 ml washing buffer (0.15 M sorbitol, 1 mM EDTA, 10 mM KH$_2$PO$_4$, pH 7.8) and homogenized with 10 (assessment of complex I) or 5 (assessment of complex III and IV) strokes. The appropriate amount of pure Percoll was added to the sample to give a final concentration of 20% (v/v). A discontinuous gradient of Percoll was cast by overlaying 5 ml of 80% (v/v) Percoll (diluted with washing buffer) with 5 ml of 33% (v/v) Percoll. A layer of 20% (v/v) Percoll was carefully added on top of the 33% (v/v) Percoll layer. The gradient was centrifuged 18,000 × g for 60 min. Mitochondria were collected at the 20-33% (v/v) interface. To remove excess Percoll, isolated mitochondria were centrifuged in washing buffer at 18,000 × g for 20 min. Pellets were resuspended in a minimal amount of washing buffer and frozen at -80 °C. All steps were performed at 4 °C.

Measuring enzymatic activity of respiratory chain complexes was performed as previously described with a Shimadzu UV mini 1240 UV-VIS spectrophotometer at 20 °C (Spinazzi et al., 2012). Concentration of mitochondrial proteins for each measurement was adjusted to 20-50 µg. The number of biological replicates was at least three.

### 2D–gel electrophoresis.

1×10$^7$ conidia of *A. fumigauss* Cea17ΔakuBKU$^{60}$ or ΔhorA were inoculated in 100 mL AMM and grown on a rotary shaker at 200 rpm and 37 °C until half of the added glucose was consumed. Mycelia were harvested through Miracloth (Calbiochem, Germany) and ground in a mortar precooled with liquid nitrogen. Cytosolic proteins of *A. fumigauss* were precipitated by adding tri-chloroacetic acid (TCA) / acetone as described previously (Kniemeyer et al.,
The pH of the samples was adjusted to pH 8.5 by titration with a 100 mM NaOH stock solution. Protein concentration was measured with the BIO-RAD protein assay (BIORAD Lab, UK) according to the Bradford method (Bradford, 1976).

The DIGE (difference in gel electrophoresis) technique was used for protein detection. Protein samples of three independent cultivations of *A. fumigatus Cea17ΔakuBKU80* and Δ*horA* were labeled with G-Dye minimal dyes (Refraction 2D-labeling kit, NHDyeAGNOSTICS, Germany) according to the manufacturer’s instructions. Briefly, 50 µg protein were labeled with 300 pmol of G-Dye fluorophores. Samples obtained from the wild type or deletion strain were labeled with either G-Dye200 or G-Dye300. For the technical replicate G-Dye labeling of the samples was reversed. A pool of all 6 samples (2 strains, 3 biological replicates) was labeled with G-Dye100 and used as internal standard. Samples were mixed and incubated for 30 min on ice. The labeling reaction was stopped with G-Dye labeling stop solution.

For 2D-gel electrophoresis, in the first dimension proteins were separated according to their isoelectric point using 24 cm strips with a non-linear pH range from both pH 3 to 7 and pH 7 to 11 (GE Healthcare Life Sciences, Germany). Isoelectric focusing, equilibration of the IPG strips and separation of the proteins in the second dimension with SDS polyacrylamide gradient gels (11 – 16 % (w/v)) was carried out as described previously (Barker *et al.*, 2012). Proteins were visualized by a Typhoon 9410 scanner (GE Healthcare Life Science, Germany) at a resolution of 100 µm. Gel images were analyzed with the Delta 2D 4.3 software (Decodon, Germany). Only spots with a ratio greater than 2 and a p-value below 0.05 using student’s t-test were regarded as significantly different. To identify differentially expressed proteins by mass spectrometry (MS), the gels were post-stained with colloidal Coomassie Brilliant Blue as described previously (Kniemeyer *et al.*, 2006) and protein spots were excised manually. Tryptic digestion of the protein spots was performed according to the
protocol of Shevchenko et al. (Shevchenko et al., 1996). Extracted peptides were measured and identified on an Ultraflex I or Ultraflextreme MALDI-TOF/TOF device using flexControl 3.3 for data collection and flexAnalysis 3.3 spectra analysis/peak list generation (Bruker Daltonics, Germany) as described previously (Vödisch et al., 2009). Peptide mass fingerprint (PMF) and peptide fragmentation fingerprint (PFF) spectra were submitted to the MASCOT server (MASCOT 2.1.03, Matrix Science, UK), searching the NCBI database limited to the taxon Fungi. Results were regarded as significant with an allowed likelihood for a random hit of $p \leq 0.05$, according to the MASCOT score. All proteome data were imported into our in-house data warehouse Omnifung (http://www.omnifung.hki-jena.de) and are publicly accessible. Identified proteins were classified by enrichment analysis using the FungiFun annotation tool (Priebe et al., 2011).

**Virulence studies.**

**Murine models.**

Established murine models for invasive pulmonary aspergillosis were used for virulence studies (Kupfah et al., 2006, Liebmann et al., 2004). Female outbreed CD-1 mice (18-20g, 6-8 week-old) were supplied by Charles River, Germany. Animals were housed under standard conditions in individually ventilated cages and fed with normal mouse chow and water ad libitum. Conidia for mice experiments were harvested from cultures grown on malt extract agar plate (Sigma-Aldrich, Germany).

In the cyclophosphamide model 140 mg $kg^{-1}$ cyclophosphamide (Sigma-Aldrich, Germany) was injected intraperitoneally on days -4, -1, 2, 5, 8 and 11 to induce leukopenia. Additionally, a single subcutaneous dose of cortisone acetate (200 mg $kg^{-1}$; Sigma-Aldrich, Germany) was applied on day -1. For the cortisone acetate model, immunosuppression was achieved by two single doses of 25 mg cortisone acetate (Sigma-Aldrich, Germany), which
were injected intraperitoneally three days before and immediately prior to infection with conidia (day 0).

For intranasal infection, mice were anesthetized by a combination of midazolam, fentanyl, and medetomidine. Under deep anesthesia, the conidia suspension was applied on both nares and inhaled. $3 \times 10^4$ conidia/mouse and $2 \times 10^5$ conidia/mouse in 20 µl PBS buffer were used in cyclophosphamide and cortisone acetate models, respectively (Liebmann et al., 2004). Anesthesia was antagonized by subcutaneous injection of flumazenil, naloxon and atipamezol.

Infected animal were monitored at least twice daily and humanely sacrificed if moribund (defined by severe lethargy, severe dyspnea, or hypothermia). Infections were performed with a group of 10 mice for each tested strain. A control group of 5 mice was mock-infected with PBS. Survival data were plotted by Kaplan–Meier curves and statistically analyzed by log rank test and Gehan–Wilcoxon test using Graph Pad Prism 5.00 (GraphPad Software, USA).

Lungs from sacrificed animals were removed, and either stored in RNAlater (Qiagen, Germany) for RNA extraction or fixed in formalin and paraffin-embedded for histopathological analyses according to standard protocols. 4-µm sections were stained using Periodic acid-Schiff (PAS, hyphae stained pink). Sections were analyzed with the Leica DMI 4000B microscope and images were taken with a Leica DFC480 camera and analyzed by Leica LAS V.3.7 software (Leica, Germany).

**Chicken embryo infection model.**

Virulence of *A. fumigatus* CEA17ΔakuB$^{KU80}$ and the deletion strains ΔhorA was tested in an established egg infection model (Jacobsen et al., 2010). Briefly, after 7 days cultivation on malt agar plates at room temperature conidia were harvested in PBS containing 0.1% (v/v) Tween 20. Conidia were diluted with PBS to a concentration of $10^4$ conidia mL$^{-1}$ just prior infection. PBS alone was used as a negative control. Embryonated eggs were incubated at
37.6 °C and 50-60% relative humidity in an incubator (BSS 300, Grumbach, Germany). After 10 days of incubation twenty eggs per group were infected with $10^3$ conidia/egg via the chorioallantoic membrane (CAM) and survival was monitored daily over 7 days by candling. Survival data were plotted as Kaplan-Meyer curves and statistically analyzed by a log rank test.

**Bioinformatic analysis.**

A phylogenetic tree of orthologous HorA protein sequences was calculated as described hereinafter. The HorA protein sequence was used to search for proteins with high sequence similarity using Blast (NCBI). Representatives of different fungal lineages (Ascomycetes, Basidiomycetes, Mucorales), bacteria, archaea, and plants were manually selected. The alignment was performed by ClustalW (Larkin *et al.*, 2007) with Gonnet protein weight matrix and otherwise standard parameters. Maximum likelihood tree were built by PhyML v3.0.1 (Guindon *et al.*, 2010) with LG model of the amino acid substitution and using aLRT (approximate likelihood-ratio test) for statistical branch support. Additional ortholog searches were performed with Phylome DB (http://phylomedb.org/) and Metaphors (http://orthology.phylomedb.org/).

The promoter region of *horA* was screened for binding sites of the transcriptional regulator SrBA. The search was accomplished by using position weight matrices (PWMs) built on motifs from two different sources: (i) binding site motifs were taken from Linde *et al.* (2012) and (ii) extracted with MEME (http://meme-suite.org/) from the CHIPseq data from Chung *et al.* (2014). The PWMs were constructed from each motif set using MEME and then used for the matrix search with RSAT (http://fungi.rsat.eu/) (Turatsinze *et al.*, 2008). The only overlapping prediction of both PWMs was considered as the most robust one and considered as SrBA transcription factor binding site.
Statistical analysis.

If not otherwise stated, the Student’s t-test was used for significance testing of two groups. Differences between the groups were considered significant if $p \leq 0.05$ or $p \leq 0.01$ were calculated. These confidence intervals are labeled with one or two asterisks, respectively. Error bars indicate standard deviations of the means.

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References


Fig. 1: A) Northern blot analysis of horA under hypoxic growth conditions. *A. fumigatus* wild type was cultivated under hypoxia (0.2 % O₂) in batch fermentation. RNA was isolated from samples taken after 0, 3, 6, 12 and 24 h of hypoxia. 18S and 28S rRNA served as loading control. B) Phylogenetic tree of HorA and 54 selected orthologs of HorA. The tree is based on ClustalX alignments of all amino acid sequences. C) Growth of *A. fumigatus* ΔhorA and horAC under normoxic and hypoxic conditions. 5 µL aliquots of each strain were spotted in a serial 10-fold dilution on AMM agar plates. Growth was documented after 72 h incubation at 37 °C under normoxic (21 % O₂) or hypoxic (0.2 % O₂) conditions. The hypoxia sensitive ΔsrbA strain was used as a control.

Fig. 2: Growth parameters of *A. fumigatus* wild type, ΔhorA and horAC in AMM. A) Germination assay. Conidia of each strain were incubated in AMM at 37 °C. The number of germlings was determined over time. B) Glucose consumption over time. 100 mL AMM were inoculated with 10⁷ conidia of each strain and incubated at 37 °C and 200 rpm. C) Growth of ΔhorA on different carbon sources. 5 µL aliquots of wild type and ΔhorA were spotted in a serial 10-fold dilution on AMM agar plates containing 1 % (w/v) casamino acids or 1 % (w/v) peptone combined with 0-20 mM glucose. Growth differences were detected after 72 h of incubation at 37 °C under normoxic conditions.

Fig. 3: Nitrosative and oxidative stress response of ΔhorA. A) Northern blot analysis of fhpA and gnoA. *A. fumigatus* wild type and ΔhorA were cultivated until half of the available glucose was consumed. 18S and 28S rRNA served as loading control. B) Inhibition zone assay with DETA-NO. 10⁶ conidia were mixed with 2 % (w/v) AMM top agar containing either 60 mM glucose or 1 % (w/v) peptone as sole carbon source. The well in the middle of the agar plate was filled with 45 µM DETA-NO. The inhibition zones were measured 16 h after incubation at 37 °C. C) Sensitivity of conidia against H₂O₂. 10⁵ conidia of *A. fumigatus* wild type, ΔhorA and horAC were incubated in the presence of 0, 0.2 and 0.4 M H₂O₂. After 30 min incubation survival was determined via a CFU assay. D) Growth of ΔhorA in the presence of menadione. 5 µL aliquots of wild type, ΔhorA and horAC were spotted in a serial 10-fold dilution on AMM agar plates in the absence or presence of 1 µM menadione. Growth differences were detected after 72 h of incubation at 37 °C under normoxic conditions. E) Northern blot analysis of horA and cat1. *A. fumigatus* wild type, ΔhorA and horAC
were cultivated in the absence or presence of 1 µM menadione until half of the available glucose was consumed. 18 S and 28 S rRNA served as loading control.

**Fig. 4:** A) Impact of reductive stress on growth of Δ*horA*. 5 µL aliquots of wild type, Δ*horA* and *horA*<sup>C</sup> were spotted in a serial 10-fold dilution on AMM agar plates containing 60 mM glucose or 1% (w/v) peptone as the sole carbon source. Reductive stress was induced by the addition of 15 mM DTT. Growth differences were detected after 48 h of incubation at 37 °C under normoxic conditions. B) Intracellular concentration of the pyridine nucleotides of *A. fumigatus* wild type, Δ*horA*, and *horA*<sup>C</sup>. Cultures with 10<sup>5</sup> conidia mL<sup>-1</sup> were incubated at 37 °C and 200 rpm until half of the available glucose was consumed. Pyridine nucleotides were extracted in PBS buffer and quantified. Left axis: pyridine nucleotide concentration [µmol/µg protein]. Right axis: ratio of NADH to NAD<sup>+</sup>. C) Subcellular localization of HorA<sub>eGFP</sub> fusion protein. The *horA<sub>eGFP</sub>* strain was cultivated in AMM for 20 h at 37 °C. Mitochondria were stained with Mitotracker Deep Red. Size bar, 10 µm.

**Fig. 5:** A) Resistance of Δ*horA* against antifungal drugs. 5 µL aliquots of wild type, Δ*horA* and *horA*<sup>C</sup> were spotted in a serial 10-fold dilution on AMM agar plates in the presence of 0.25 µg mL<sup>-1</sup> voriconazole, 0.5 µg mL<sup>-1</sup> terbinafine and 5 µg mL<sup>-1</sup> amphotericin B. Growth differences were detected after 72 h (120 h for amphotericin B) of incubation at 37 °C under normoxic conditions.

**Fig. 6:** Virulence of *A. fumigatus* wild type, Δ*horA* and *horA*<sup>C</sup>. A) Cortisone acetate model. Groups of 10 female outbreed CD-1 mice were infected with 2×10<sup>5</sup> conidia each by nasal inhalation; B) Histopathology of representative sections of lungs from mice infected with *A. fumigatus* conidia from wild type (WT), Δ*horA* and *horA*<sup>C</sup>. Sections of lungs from several mice infected with the Δ*horA* mutant strain are shown. C) Cyclophosphamide model. Groups of 10 female outbreed CD-1 mice were infected with 3×10<sup>4</sup> conidia each by nasal inhalation; D) Histopathology of representative sections of lungs from mice infected with *A. fumigatus* conidia from wild type (WT), Δ*horA* and *horA*<sup>C</sup>. Sections of lungs from several mice infected with the Δ*horA* mutant strain are shown. Survival was monitored for 14 days. A group of mice received PBS and served as mock infected control.
Table 1: Quantity of extracted coenzyme Q10 from *A. fumigatus* mitochondria comparing the wild type to ∆horA

<table>
<thead>
<tr>
<th><em>A. fumigatus</em> strain</th>
<th>µg Q10 /mg of mitochondria</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wild type</td>
<td>3.19±1.34</td>
</tr>
<tr>
<td>∆horA</td>
<td>0.15±0.01</td>
</tr>
</tbody>
</table>

Table 2: Specific enzymatic activity of mitochondrial respiratory complexes in *A. fumigatus* wild type and the mutant strain ∆horA\(^1\)

<table>
<thead>
<tr>
<th>RC enzyme complex</th>
<th>Complex I</th>
<th>Alternative NADH:ubiquinone oxidoreductase(^2)</th>
<th>Complex III</th>
<th>Complex IV</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wild type</td>
<td>226.0±48.0</td>
<td>520.6±50.1</td>
<td>372.1±88.5</td>
<td>263.5±95.0</td>
</tr>
<tr>
<td>∆horA</td>
<td>39.8±29.0</td>
<td>730.6±84.5</td>
<td>289.3±22.3</td>
<td>567.0±98.2</td>
</tr>
</tbody>
</table>

\(^1\)Specific enzyme activity of each mitochondrial respiratory complex given in nmol min\(^{-1}\) mg\(^{-1}\) of total protein.

\(^2\)Rotenone-resistant activity
Fig. 1: A) Northern blot analysis of horA under hypoxic growth conditions. *A. fumigatus* wild type was cultivated under hypoxia (0.2 % O$_2$) in batch fermentation. RNA was isolated from samples taken after 0, 3, 6, 12 and 24 h of hypoxia. 18S and 28S rRNA served as loading control. B) Phylogenetic tree of HorA and 54 selected orthologs of HorA. The tree is based on ClustalX alignments of all amino acid sequences. C) Growth of *A. fumigatus* ΔhorA and horAC under normoxic and hypoxic conditions. 5 µL aliquots of each strain were spotted in a serial 10-fold dilution on AMM agar plates. Growth was documented after 72 h incubation at 37 °C under normoxic (21 % O$_2$) or hypoxic (0.2 % O$_2$) conditions. The hypoxia sensitive ΔsrbA strain was used as a control.
Fig. 2: Growth parameters of *A. fumigatus* wild type, ΔhorA and *horA*<sup>C</sup> in AMM. A) Germination assay. Conidia of each strain were incubated in AMM at 37 °C. The number of germlings was determined over time. B) Glucose consumption over time. 100 mL AMM were inoculated with 10<sup>7</sup> conidia of each strain and incubated at 37 °C and 200 rpm. C) Growth of ΔhorA on different carbon sources. 5 µL aliquots of wild type and ΔhorA were spotted in a serial 10-fold dilution on AMM agar plates containing 1 % (w/v) casamino acids or 1 % (w/v) peptone combined with 0-20 mM glucose. Growth differences were detected after 72 h of incubation at 37 °C under normoxic conditions.
Fig. 3: Nitrosative and oxidative stress response of \( \Delta \text{horA} \). A) Northern blot analysis of \( \text{fhpA} \) and \( \text{gnoA} \). \( \text{A. fumigatus} \) wild type and \( \Delta \text{horA} \) were cultivated until half of the available glucose was consumed. 18S and 28S rRNA served as loading control. B) Inhibition zone assay with DETA-NO. \( 10^8 \) conidia were mixed with 2\%(w/v) AMM top agar containing either 60 mM glucose or 1\%(w/v) peptone as sole carbon source. The well in the middle of the agar plate was filled with 45 \( \mu \text{M} \) DETA-NO. The inhibition zones were measured 16 h after incubation at 37 °C. C) Sensitivity of conidia against \( \text{H}_2\text{O}_2 \). \( 10^5 \) conidia of \( \text{A. fumigatus} \) wild type, \( \Delta \text{horA} \) and \( \text{horA}^C \) were incubated in the presence of 0, 0.2 and 0.4 M \( \text{H}_2\text{O}_2 \). After 30 min incubation survival was determined via a CFU assay. D) Growth of \( \Delta \text{horA} \) in the presence of menadione. 5 \( \mu \text{L} \) aliquots of wild type, \( \Delta \text{horA} \) and \( \text{horA}^C \) were spotted in a serial 10-fold dilution on AMM agar plates in the absence or presence of 1 \( \mu \text{M} \) menadione. Growth differences were detected after 72 h of incubation at 37 °C under normoxic conditions. E) Northern blot analysis of \( \text{horA} \) and \( \text{cat1} \). \( \text{A. fumigatus} \) wild type, \( \Delta \text{horA} \) and \( \text{horA}^C \) were cultivated in the absence or presence of 1 \( \mu \text{M} \) menadione until half of the available glucose was consumed. 18 S and 28 S rRNA served as loading control.
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Here we characterized the fungus-specific mitochondrial oxidoreductase HorA in *Aspergillus fumigatus*. HorA is involved in the coenzyme Q biosynthesis and consequently important for function of respiratory chain complex I and redox homeostasis. Our results indicate that HorA is crucial for virulence and drug tolerance of *A. fumigatus*. Moreover, HorA conceptionally provides an attractive mitochondrial target for therapy of fungal infections due to its absence in mammals.