Aspergillus fumigatus Copper Export Machinery and Reactive Oxygen Intermediate Defense Counter Host Copper-Mediated Oxidative Antimicrobial Offense

Graphical Abstract

Highlights

- Aspergillus fumigatus infection activates the host copper (Cu) transporter Ctr1
- AceA is the A. fumigatus transcription factor coordinating Cu-dependent defense
- A. fumigatus detoxifies high copper levels through the P-type ATPase CrpA
- Activation of copper export restores virulence of aceA-deficient strains

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In Brief
Wiemann et al. find that Aspergillus fumigatus employs the copper-sensing transcription factor AceA to express the copper exporter CrpA as a defense mechanism against macrophages. Copper and reactive oxygen intermediate attack and defense are inextricably connected on the side of both host and pathogen during infection.
Aspergillus fumigatus Copper Export Machinery and Reactive Oxygen Intermediate Defense Counter Host Copper-Mediated Oxidative Antimicrobial Offense

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SUMMARY

The Fenton-chemistry-generating properties of copper ions are considered a potent phagolysosome defense against pathogenic microbes, yet our understanding of underlying host/microbe dynamics remains unclear. We address this issue in invasive aspergillosis and demonstrate that host and fungal responses inextricably connect copper and reactive oxygen intermediate (ROI) mechanisms. Loss of the copper-binding transcription factor AceA yields an Aspergillus fumigatus strain displaying increased sensitivity to copper and ROI in vitro, increased intracellular copper concentrations, decreased survival in challenge with murine alveolar macrophages (AMs), and reduced virulence in a non-neutropenic murine model. ΔaceA survival is remediated by dampening of host ROI (chemically or genetically) or enhancement of copper-exporting activity (Cra) in A. fumigatus. Our study exposes a complex host/microbe multifactorial interplay that highlights the importance of host immune status and reveals key targetable A. fumigatus counter-defenses.

INTRODUCTION

The ubiquitous, saprophytic mold Aspergillus fumigatus forms and releases asexual airborne spores (conidia) (Latgé, 1999). In the immunocompetent individual, inhalation of conidia does not usually cause disease, as professional phagocytes such as alveolar macrophages (AMs) and neutrophils prevent the development of aspergillosis (Dagenais and Keller, 2009; Gilbert et al., 2014; Heinekamp et al., 2015). However, a spectrum of immune deficiencies in the population render patients susceptible to invasive growth. The first line of defense is phagocytosis of inhaled conidia by AMs and neutrophils. AMs reside beneath the alveolar surfactant film where they represent 90% of the resident leukocytes in the lung (Hasenberg et al., 2011). Molecular mechanisms by which AMs and neutrophils destroy inhaled A. fumigatus spores are only partially understood. Together, these data imply that transition metal homeostasis (mainly iron, copper, and zinc) and production of reactive oxygen intermediates (ROIs) are the major strategies employed to kill A. fumigatus conidia (Clark et al., 2016; Dagenais and Keller, 2009; Heinekamp et al., 2015; Kasa-hara et al., 2016; Lanternier et al., 2013; Park and Mehrad, 2009).

Accumulating evidence suggests that innate phagocyte defense not only includes toxic ROIs generated through the phagocyte NADPH oxidase (PHOX) complex but also utilizes copper as a microbial toxin (Ding et al., 2014; Djoko et al., 2015; Garcia-Santamaria and Thiele, 2015). Similar to iron, copper is a Janus-faced transition metal functioning on the one hand as an essential cofactor for enzymes like cytochrome c oxidase (complex IV), superoxide dismutases (SODs), laccases, and reductive iron transporters, and on the other hand as a catalyst in toxic ROI-generating Fenton chemistry. Infection studies with Mycobacterium species, Salmonella typhimurium, and Cryptococcus neoformans suggest that macrophages elevate copper levels inside the phagosome by increasing expression of the copper importer Crt1 and locating the P-type copper ATPase pump (ATP7A) to the phagosomal membrane (Achard et al., 2012; Ding et al., 2013; White et al., 2009).

Fungi utilize several protein classes to regulate copper homeostasis, including copper-binding transcription factors, copper transporters (import and export), and copper-binding...
metallothioneins (Table 1). Copper-binding transcription factors ensure correct expression of genes required for survival in insufficient or toxic copper environments. In Saccharomyces cerevisiae, copper deficiency is sensed by the copper-binding transcription factor Mac1p that, in the absence of copper, activates the plasma membrane-localized copper transporters Ctr1p and Ctr3p as well as Fre1p, a metalloreductase that mobilizes copper ions from oxidized copper complexes (Cyert and Philpott, 2013; Graden and Winge, 1997; Jungmann et al., 2004). Copper excess is sensed by the copper-binding transcription factor Ace1p (also called Cup2p), which activates expression of the metallothionein-encoding genes CUP1 and CRS5 (Culotta et al., 1994; Ecker et al., 1986; Thiele, 1988). In addition, Ace1p induces SOD1 (encoding a copper-dependent SOD) and metalloreductase-encoding genes (FREs) (Cyert and Philpott, 2013).

Human pathogenic fungi follow suit with deviations dependent on species. Physiological studies of the pathogenic ascomycete Candida albicans identified a putative homolog of the human ATP7A P-type copper ATPase and S. cerevisiae Ccc2p (Lowe et al., 2004), Crp1p, as critical for copper detoxification with the metallothionein Cup1p responsible for residual copper resistance when CRP1 was deleted and both proteins essential for establishing full virulence (Douglas et al., 2011; Mackie et al., 2016; Schwartz et al., 2013; Weissman et al., 2000) (Table 1). Both CRP1 and CUP1 are induced by elevated copper concentrations through the homolog of Ace1p (Schwartz et al., 2013; Weissman et al., 2000). In the pathogenic basidiomycete C. neoformans, one copper-binding transcription factor, Cuf1, regulates expression of both copper importers Ctr1 and Ctr4 as well as the two metallothioneins Cmt1 and Cmt2 involved in copper detoxification (Ding et al., 2011; Waterman et al., 2007). Deletion of either cuf1 or cmt1/cmt2 results in attenuated virulence of C. neoformans. The copper transporter ctr4 in C. neoformans is essential for establishing full virulence during meningoencephalitis rather than pulmonary infection (Ding et al., 2013; Sun et al., 2014; Waterman et al., 2007, 2012).

Little is known about copper homeostasis in A. fumigatus. This opportunistic human pathogen encodes four putative copper importers (CtrA1, CtrA2, CtrB, and CtrC) (Table 1) (Park et al., 2014). A double deletion mutant of ctrA2 and ctrC showed reduced SOD and catalase activities but was not altered in virulence in an immunocompromised murine model of invasive aspergillosis (IA) (Park et al., 2014). Complicating an understanding of A. fumigatus virulence factors is the growing realization that host immune status often dictates IA progression. Neutropenic and non-neutropenic populations are both susceptible to IA (Russo et al., 2011), and murine models of these two conditions can display differential outcomes. This is illustrated in a compilation of five studies showing gliotoxin to be a virulence factor only in the murine non-neutropenic IA model (Dagenais and Keller, 2009). Furthermore, some inherited primary immunodeficiencies such as chronic granulomatous disease (CGD), which lack the ROI-generating leukocyte NADPH oxidase, are highly associated with IA development (Lanternier et al., 2013).

Because to date there is no information on how A. fumigatus regulates genes involved in copper acquisition and detoxification, we set out to identify copper-dependent regulators and characterize their role in IA progression. We also assessed the importance of copper-mediated defense in a wide breadth of host immune status capabilities using multiple host IA models. We reveal the inextricable interface of copper and ROI...

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**Table 1. Relevant Copper-Binding Proteins in A. fumigatus, S. cerevisiae, C. albicans, and C. neoformans**

<table>
<thead>
<tr>
<th>ID</th>
<th>Name</th>
<th>S. cerevisiae</th>
<th>C. albicans</th>
<th>C. neoformans</th>
<th>Description</th>
</tr>
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<tbody>
<tr>
<td>AfuA_6G07780</td>
<td>AceA</td>
<td>Ace1p (Cup2p)</td>
<td>Cup2</td>
<td>copper-toxicity TF</td>
<td></td>
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<tr>
<td>AfuA_1G13190</td>
<td>MacA</td>
<td>Mac1p (Cua1p)</td>
<td>Mac1</td>
<td>copper-starvation TF</td>
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<tr>
<td>AfuA_2G01190</td>
<td>CufA</td>
<td>Haa1p</td>
<td></td>
<td>dual-function copper-binding TF</td>
<td></td>
</tr>
<tr>
<td>AfuA_6G02810</td>
<td>CtrA2</td>
<td>Ctr1p</td>
<td>Ctr1</td>
<td>high-affinity copper transporter</td>
<td></td>
</tr>
<tr>
<td>AfuA_2G03730</td>
<td>CtrC</td>
<td>Ctr3p</td>
<td>Ctr4</td>
<td>high-affinity copper transporter</td>
<td></td>
</tr>
<tr>
<td>AfuA_3G08180</td>
<td>Ctr2</td>
<td>Ctr2p</td>
<td>Ctr2</td>
<td>low-affinity copper transporter</td>
<td></td>
</tr>
<tr>
<td>AfuA_3G13660</td>
<td>CtrA1</td>
<td>Ctr1</td>
<td></td>
<td>unknown function</td>
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<tr>
<td>AfuA_4G12620</td>
<td>CptA</td>
<td>Ccc2p</td>
<td>Ccc2</td>
<td>intracellular copper ATPase</td>
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<tr>
<td>AfuA_3G12740</td>
<td>CrpA</td>
<td>Crp1</td>
<td></td>
<td>copper-exporting ATPase</td>
<td></td>
</tr>
<tr>
<td>AfuA_4G04318</td>
<td>CmtA</td>
<td>Cup1p</td>
<td>Cup1</td>
<td>copper metallothioneins</td>
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<tr>
<td>AfuA_5G09240</td>
<td>Sod1</td>
<td>Sod1p</td>
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<tr>
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<td>Sod1p</td>
<td>Sod1</td>
<td>copper metallothioneins</td>
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</tr>
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</table>

*Park et al., 2014.
Upadhay et al., 2013.
Lambou et al., 2010.*
mechanisms in both host and microbe and demonstrate that host copper dynamics potentiate ROI stress for A. fumigatus. The copper-binding transcription factor AceA is a virulence factor in a non-neutrophenic IA model. Our biochemical and virulence data strongly support a mechanism of an inability of A. fumigatus to manage host-derived copper imported by host copper ion transporters. This macrophage sensitivity is corrected by either AceA regain of activity of the putative copper exporter CrpA or the spore-specific ROI response bZIP protein AtfA. Furthermore, the inability of the host to mount an ROI defense dampens a copper defense response as demonstrated by equivalent persistence of AceA to that of wild-type A. fumigatus in both zebrafish and murine PHOX-deficient hosts.

RESULTS

The Genome of Aspergillus fumigatus Encodes Three Putative Copper-Binding Factors

Our interest in copper regulation was originally piqued by microarray data in which a putative copper-binding transcription factor encoding gene (AfuA_6G07780) was among the most downregulated transcription factor genes in the reduced virulence JlaeA mutant (Perrin et al., 2007). We next identified all proteins encoded in the genome that harbor a conserved copper-fist DNA-binding domain (Jungmann et al., 1993; Szczypka and Thiele, 1988). A domain search in the A. fumigatus AF293 genome database (Cerqueira et al., 2014) using the conserved copper-fist DNA-binding domain C-X2-C-X8-C-X-H (InterPro ID: IPR001083) resulted in two additional hits (AfuA_1G13190 and AfuA_2G01190). Protein alignment using the three A. fumigatus sequences and characterized copper-binding transcription factor sequences from S. cerevisiae and other fungi showed that AfuA_1G13190 groups with the nutritional copper-binding transcription factors including Mac1p from S. cerevisiae and is most closely related to GRISEA and other fungi showed that AfuA_1G13190 groups with the nutritional copper-binding transcription factors including Mac1p from S. cerevisiae and is most closely related to GRISEA. AfuA_2G01190 contains a cysteine-rich motif in its C-terminus which was most closely related to GR1SEA from the filamentous ascomycete Podospora anserina (Borghouts and Osiewacz, 1998) and was therefore assigned the name MacA (Figure S1A). Unexpectedly, AfuA_2G01190 and AfuA_6G07780 also group to the Mac1 family and within this group are closest to Cuf1 from C. neoformans (Figure S1A) (Ding et al., 2019). Yeast copper-binding transcription factors involved in copper detoxification including Crf1, Amt1, Cup2, and Cup2p/Ace1p from Yarrowia lipolytica, Candida glabrata, C. albicans, and S. cerevisiae form a distinct group of related proteins (Figure S1A).

As it was not obvious from phylogeny alone whether AfuA_2G01190 or AfuA_6G07780 more likely regulates copper toxicity, we examined all three proteins in detail for predicted copper regulatory motifs. In addition to the N-terminally located conserved copper-fist Zn(II)- and DNA-binding motif found in all three proteins, MacA/AfuA_1G13190 contains a cysteine-rich motif in its C terminus that aligns with the cysteine-rich C2 motif of Mac1p (Figure S1B) known to be involved in inactivation of the protein under replete copper conditions in S. cerevisiae (Graden and Winge, 1997; Jensen and Winge, 1998; Keller et al., 2000). The protein sequences of AfuA_2G01190 and AfuA_6G07780 are missing this C-terminally located motif but contain additional cysteine residues in their respective N termini in proximity to the copper-fist DNA-binding domain (Figure S1B). Of these, AfuA_6G07780 contains all eight cysteine residues required for Ace1p functionality in S. cerevisiae (Hu et al., 1990) and was therefore assigned the name AceA. AfuA_2G01190 is missing four cysteine residues and was named CufA (Figure S1B). This finding is reminiscent of S. cerevisiae Haa1p, which has significant homology to Ace1p but is lacking one of the eight conserved cysteine residues (Figure S1).

Copper Detoxification by AceA Relieves ROI Stress

To test whether and how the identified copper-fist DNA-binding domain proteins in A. fumigatus affect copper homeostasis in growth studies, we constructed gene deletion mutants of each gene, and—due to phenotypes described below—additionally complemented the ΔaceA mutant with a wild-type gene copy (Figure S2A). The sensitivity of ΔaceA to copper became apparent at 5 μM copper on solidified growth media after 2 days (Figures 1A and 1B). This hypersensitivity of the ΔaceA mutant is specific to copper ions, as addition of 100 μM Cd or Fe in copper-depleted medium revealed no growth difference between the ΔaceA and wild-type strain (Figure S2B). Complementation of ΔaceA with a wild-type aceA copy restored normal growth (Figure S2A). The ΔmacA and ΔcufA strains exhibited milder phenotypes with ΔmacA forming fewer and non-pigmented spores in copper depletion conditions (Figure 1; Figure S2C). However, when the copper chelator, bathocuproinedisulfonic acid, was added to the medium, the ΔmacA strain showed very sick growth (Figure S2C). Similarly, when media were prepared with trace elements containing the metal ion chelator EDTA, the ΔmacA strain showed a severe growth reduction on media even when 5 μM copper was added (Figure S2D). Using the same EDTA-containing media, 50 μM copper did not cause any growth reduction of the WT or the ΔaceA strain (Figure S2D). Together, these latter results highlight the importance of fungal growth conditions for experimentation.

Because on the one hand copper is involved in detoxification of superoxide (O2−) as a cofactor of copper-dependent SODs and on the other hand can contribute to hydroxy radical (‘OH) production from hydrogen peroxide (H2O2) by participation in Fenton chemistry, we tested the mutants for synergistic effects of increasing copper and the intracellular O2− generator, menadione (Thor et al., 1982; White and Clark, 1988). When we grew the strains on increasing copper concentrations and 2 μM menadione, we observed a synergistic growth-inhibitory effect for all strains that was most severe in the ΔaceA mutant (Figure 1C). When we assessed the sensitivities of the strains toward H2O2 under increasing copper concentrations, we observed the same trend with an even more severe inhibition of growth of the ΔaceA strain (Figure S2F). This copper-dependent growth defect could be alleviated when the reducing agent reduced L-glutathione (GSH) was added to high copper-containing media in all strains (Figure 1C; Figures S2A and S2E), suggesting that copper increases ROI stress in an AceA-dependent fashion.

AceA Contributions to Host Infection

Because A. fumigatus encounters AMs as one of the first lines of host defense, we compared the survival rates of the wild type to the ΔmacA, ΔcufA, and ΔaceA mutants in murine AMs.
When challenged with macrophages, the wild type and the \( \Delta \text{macA} \) strain displayed a survival rate of ~25%, whereas the \( \Delta \text{aceA} \) mutant only showed ~10% survival (Figure 2A). Interestingly, deletion of \( \text{cufA} \) increased survival of spores when challenged with macrophages (Figure 2A). Next, infection assays were performed using both a non-neutropenic (cortisone acetate) and neutropenic (cyclophosphamide) murine model of IA. The \( \Delta \text{aceA} \) mutant was significantly less virulent than the wild type and reconstituted strains in the non-neutropenic model (Figure 2B). In line with the reduced virulence, the \( \Delta \text{aceA} \) mutant formed less numerous and smaller infection loci compared to the wild type in the infected lung tissue, as assessed by histopathology and colony-forming unit (CFU) enumeration (Figures S3 A and S3B). Levels of TNF-alpha in the lungs showed no differences between the two strains, although they were significantly higher than in uninfected mice (Figure S3C). Similar to the assays performed with murine AMFs, the \( \Delta \text{macA} \) mutant showed wild-type-like virulence; however, despite the elevated survival rate in the macrophage assay, the \( \Delta \text{cufA} \) strain did not show increased virulence in this model (Figure 2C). Although not significant compared to wild type, analysis of the \( \Delta \text{aceA} \) strain in the neutropenic IA model presented ambiguous results considering the p value (\( p = 0.0662 \)) and its decreased virulence in comparison to the complemented control (\( p = 0.0008 \)) (Figure 2D). There was no difference in virulence between \( \Delta \text{cufA} \) or \( \Delta \text{macA} \) and wild type in this model (Figure 2E).

**Macrophage Copper Flux Is Altered in \( \Delta \text{aceA} \) Cells**

Activation of macrophage ATP7A copper ATPase coupled with the importer Crt1 are implicated in host-mediated copper accumulation in the phagosome during bacterial infections (Wagner et al., 2005; White et al., 2009). Furthermore, murine infections with \( C. \text{neoformans} \) increased serum copper levels and altered expression of both ATP7A and Ctr1 in murine bronchoalveolar lung cells (Ding et al., 2013). Thus, we reasoned that activity of this conserved defense response could also be induced by \( A. \text{fumigatus} \) infection and measurable in copper levels in macrophage confrontations between wild-type and \( \Delta \text{aceA} \) \( A. \text{fumigatus} \) strains.

We first examined for any alterations in ATP7A or Ctr1 dynamics. Western blot analysis of non-infected and \( A. \text{fumigatus} \)-challenged granulocyte-macrophage colony-stimulating factor (GM-CSF)-activated bone marrow-derived murine macrophages (BMDMs) showed a significant induction of Ctr1 of challenged cells compared to non-infected cells (Figure 3 A; Figures S3 D and S3E). Immunohistochemistry analysis of the murine copper ATPase ATP7A showed an increased fluorescent signal in \( A. \text{fumigatus} \)-challenged BMDMs that showed aggregation in distinct foci, sometimes distinctly surrounding fungal spores. These signals are distinctively different than the signals observed in non-challenged cells (Figure 3B).

Next, we determined total copper levels in \( A. \text{fumigatus} \) spores (wild-type, \( \Delta \text{aceA} \), and \( \text{aceA}^{\Delta} \) strains) either unchallenged or challenged with GM-CSF-activated BMDMs using inductively coupled plasma mass spectrometry (ICP-MS) (Subramanian Vignesh et al., 2013). Total copper, zinc, and iron quantification was also carried out in BMDMs incubated with the \( A. \text{fumigatus} \) strains. As demonstrated with AMFs (Figure 2), the \( \Delta \text{aceA} \) mutant had a lower survival rate in BMDMs (Figure 3C). Quantification of total copper ion levels in spores challenged with BMDMs showed
an increased copper concentration in ΔaceA spores compared to unchallenged ΔaceA spores (Figure 3D). This increase did not occur in wild-type and reconstituted aceA strains (Figure 3D). Quantification of the copper content in macrophages challenged with the different strains showed that the cells incubated with ΔaceA spores had a decreased total copper concentration, suggesting a mobilization of copper to the ΔaceA spores—a trend that was not observed for the wild type or the reconstituted aceA strain (Figure 3E). Importantly, the level of zinc or iron in macrophages incubated with the ΔaceA spores was not decreased relative to the wild-type and aceA⁵ strains (Figures S3F and S3G).

Together, these data strongly support copper mobilization to fungal tissue as one means of defense. To further examine a role for ATP7A in IA progression of wild-type and ΔaceA strains, we compared fungal burden in both immunocompetent and ATP7A-deficient zebrafish larvae using our previously established zebrafish IA model (Knox et al., 2014; Mendelsohn et al., 2006). The larval zebrafish has functionally conserved and competent vertebrate innate immune mechanisms (Harvie and Huttenlocher, 2015; Herbomel et al., 1999; Le Guyader et al., 2008), and previous studies have demonstrated the conserved nature of zebrafish ATP7A to the mammalian ortholog (Madsen et al., 2008). Although we saw a significant increase in wild-type burden in the ATP7A morphants (Figure 3F), there was no rescue of wild-type-like growth in the ΔaceA strain in the ATP7A-deficient zebrafish. However, addition of the copper chelator ammonium tetrathiomolybdate (TTM) (Brewer, 2005) showed a similar restoration of ΔaceA survival to wild-type-like levels that were significantly higher than in the untreated ΔaceA infection (Figure 3G).

Depleting Host ROI Synthesis Remediates ΔaceA Survival in Host Tissues

Considering that the ΔaceA strain grew poorly in the ATP7A-deficient larval zebrafish and is sensitive to ROI, we considered an alternative host mechanism in addressing the ΔaceA phenotype. Because macrophages deploy mechanisms of O₂⁻/C⁰ production by the PHOX complex to fight pathogens (Hogan and Wheeler, 2014; Lambeth and Neish, 2014) and our physiological studies (Figure 1C; Figure S2) suggest a copper-dependent ROI-sensitivity of the ΔaceA strain, we asked whether dampening ROI stress would restore ΔaceA survival in AMFs.

To test whether inhibition of O₂⁻ production by host immune cells and/or copper limitation by chemical chelation would restore wild-type-like survival rates of the ΔaceA mutant, we performed three experiments. First, we used the pharmacological PHOX complex inhibitor diphosphenyleneiodonium (DPI) (O’Donnell et al., 1993; Philippe et al., 2003) in our AMF experiment and observed that survival of the ΔaceA strain returned to
wild-type levels (Figure 4A). Second, in an in vivo complementary approach, we compared fungal burden in both immunocompetent and p22phox-deficient zebrafish larvae (Knox et al., 2014; Tauzin et al., 2014). The larval zebrafish has been used to study PHOX activity during C. albicans infection (Brothers et al., 2011), highlighting conserved ROI-generating pathways in this model (Niethammer et al., 2009). Examining wild-type and DaceA persistence in whole-larval homogenates revealed that attenuated DaceA survival was dependent on p22phox expression (Figure 4B). Third, we compared fungal burden of the A. fumigatus wild type and the DaceA mutant in a murine model (p91phox-deficient) of CGD and observed a significant increase of fungal burden of both strains in CGD mice compared to immunocompetent mice (Figure 4C; Figure S3L). In contrast to the reduced virulence and fungal burden of the ΔaceA mutant compared to the wild type that we observed in our immunocompromised murine infection model (Figure 2B; Figures S3A–S3C), both strains showed no significant difference in fungal burden in CGD mice (Figure 4C; Figure S3L).

AceA Transcriptionally Regulates Copper and ROI Detoxification Genes

The susceptibility of the ΔaceA strain to copper and ROI exposure (Figures 2 and 4), supported a role for AceA in regulating genes involved in both copper and ROI detoxification. We assessed such a possibility by examining gene expression in

Figure 3. ΔaceA Strains Accumulate More Copper during Macrophage Encounters
(A) Western blot against mouse Ctr1 and GAPDH of murine bone marrow-derived macrophages activated with GM-CSF that were unchallenged or challenged with A. fumigatus spores for 2 hr.
(B) Immuno-staining against mouse ATP7A of murine bone marrow-derived macrophages activated with GM-CSF that were unchallenged or challenged with A. fumigatus spores for 2 hr. Scale bars represent 10 μM.
(C) CFUs of fungal strains after incubation with murine bone marrow-derived macrophages activated with GM-CSF for 2 hr. Experiments were carried out in triplicates; error bars represent SDs, and statistical significance is indicated by p values.
(D) Total copper concentration of unchallenged 3 x 10^6 spores (solid) and 3 x 10^6 spores incubated with 1 x 10^7 GM-CSF-activated BMDMs for 2 hr. Experiments were carried out in triplicates; error bars represent SDs, and statistical significance is indicated by p values.
(E) Total copper concentration of 1 x 10^7 GM-CSF-activated BMDMs incubated with 3 x 10^7 spores of the indicated A. fumigatus strains for 2 hr. Experiments were carried out in triplicates; error bars represent SDs, and statistical significance is indicated by p values.
(F) CFU of fungal strains from whole zebrafish larvae at 24 hr post-microinjection. Genetic inhibition of ATP7A was obtained with morpholino-mediated knockdown (ATP7AMO). Data shown are pooled from four independent experimental replicates where significance is indicated by p values as determined by a least-squares means analysis.
(G) CFUs of fungal strains after incubation with murine AMFs supplemented with or without 50 μM tetrathiomolybdate (TTM) for 2 hr. Experiments were carried out in triplicates; error bars represent SDs, and statistical significance is indicated by p values.
both wild type and \( \Delta \text{aceA} \) in copper-depleted and excess conditions. We tested expression of the four copper importers identified in \( A. \text{fumigatus} \) (Table 1) as well as genes implicated in copper detoxification. Search of the \( A. \text{fumigatus} \) genome for putative homologs of the \( C. \text{albicans} \) copper exporter-encoding gene \( \text{crp1} \) and the \( S. \text{cerevisiae} \) copper metallothioneins \( \text{CUP1} \) and \( \text{CRS5} \) identified one homolog each that we call \( \text{crpA} \) (G12740) and \( \text{cmtA} \) (AfuA 4G04318), respectively (Table 1; Figure S4A). We also tested for the expression of the copper-dependent SOD \( \text{sod1} \) and the two mycelial catalases \( \text{cat1} \) and \( \text{cat2} \) as well as the spore catalase \( \text{catA} \).

Our results show that all four copper importers, \( \text{ctrA1} \), \( \text{ctrA2} \), \( \text{ctrB} \), and \( \text{ctrC} \), are induced under copper-depleted conditions (Figure 5A; Figure S4B). We observed an induction of \( \text{sod1} \), \( \text{cat1} \), and \( \text{cat2} \) (\( \text{catA} \) was not detectable) by copper addition with \( \text{cat1} \) and \( \text{cat2} \) also regulated by AceA (Figure 5A). Additionally, we found that the ROI-responsive transcription factor \( \text{atfA} \) and \( \text{yap1} \) were slightly induced under copper surplus conditions in an AceA-dependent manner (Figure S4C). Under the conditions tested, no signal for \( \text{cmtA} \) was detected (data not shown), whereas \( \text{crpA} \) was highly induced by copper addition in an AceA-dependent manner (Figure 5A).

The Putative Copper-Exporting P-Type ATPase \( \text{CrpA} \) and Spore-Specific ROI Defense bZIP Transcription Factor \( \text{AtfA} \) Mediate \( \Delta \text{aceA} \) Macrophage Survival

The transcription profiling (Figure 5A; Figures S4B and S4C) suggested that both ROI degradation pathways and copper export could be contributing to \( \Delta \text{aceA} \) phenotype. To test the former hypothesis, we investigated whether constitutive expression of the \( A. \text{fumigatus} \) bZIP transcription factor-encoding gene \( \text{atfA} \), which is known for its involvement in spore maturation and spore ROI defense (Hagiwara et al., 2009, 2014, 2016), could restore the \( \Delta \text{aceA} \) survival defect in macrophages (Figure 5B). As previously reported (Pereira Silva et al., 2017), we observed a significant loss of survival in activated BMDMs challenged with an \( \Delta \text{atfA} \) mutant compared to the wild type that was similar to the \( \Delta \text{aceA} \) strain (Figure 5B). Forced expression of \( \text{atfA} \) brought survival back to wild-type levels in a \( \Delta \text{aceA} \) background (Figure 5B) despite its poor growth phenotype when grown on solidified media (Figure S5A). Because \( \text{AtfA} \) is suggested to specifically govern spore ROI defense, we tested spore sensitivity toward \( \text{H}_2\text{O}_2 \) with 5 \( \mu \)M copper present and observed a significant
reduction in CFUs of the ΔattA and ΔaceA strain compared to the wild type (Figure S5B). When attA was overexpressed in the ΔaceA background, spore viability was significantly increased (Figure S5B).

Next, we deleted cmtA, crpA, and constitutively expressed crpA in both a wild-type and ΔaceA background (Figure S5C). Phenotypic analysis on growth media with elevated copper concentrations demonstrated that deletion of cmtA did not affect the growth on elevated copper conditions nor survival when the strain was challenged with murine AMFs (Figures S5C and S5D). In contrast, deletion of crpA resulted in hypersensitivity to copper compared to the wild type and ΔaceA strain (Figure S5C). When crpA is constitutively expressed, copper tolerance exceeds the wild type in both an aceA-sufficient and -deficient background (Figure S5E). Quantification of copper in mycelia grown in copper-depleted submerged conditions showed no significant difference between the wild type and the ΔcrpA strain (Figure S5H). However, spores collected from solidified media containing 5 μM copper showed a significant increase in copper of the ΔcrpA strain compared to the wild type (Figure S5H).

**DISCUSSION**

Copper has been suggested to play a major role in innate immune functions against prokaryotic and eukaryotic microbial pathogens (Ding et al., 2014; Djoko et al., 2015; Festa and Thiele, 2012; García-Santamarina and Thiele, 2015; Hodgkinson and...
Petris, 2012). Studies with bacterial and yeast pathogens have shown that phagocytes isolated from hypocupremic conditions displayed reduced phagocytotic and antimicrobial activities (Babu and Failla, 1990; Heresi et al., 1985; Xin et al., 1991). In line with these findings, copper pretreatment of phagocytes enhanced intracellular killing of Escherichia coli (White et al., 2009), and copper chelation with a non-permeable chelator increased intracellular survival of Salmonella enterica (Achard et al., 2012). Several studies involving the ascomycete pathogen C. albicans and basidiomycete C. neoformans clearly demonstrate the importance of copper-mediated phagocytic killing of these yeasts (Ding et al., 2013; Mackie et al., 2016). Although our experimentation with the filamentous fungus A. fumigatus confirms the importance of this host defense mechanism, our work exposes the limitations of copper-mediated defense and reveals the inextricable involvement of both host ROI defense and ROI countermeasures in Aspergillus (Figure 6).

Host Mechanisms and Immune Status Underlies Importance of Copper-Mediated Defense in IA

Although the precise mechanisms of phagocyte copper mobilization remain to be fully explored, studies in E. coli and S. enterica (Achard et al., 2012; White et al., 2009) and recent work on C. neoformans (Ding et al., 2013) have demonstrated that phagocytes respond with upregulation of CTR1 and ATP7A (White et al., 2009). Similarly, we have shown here that macrophages encountering A. fumigatus spores react by upregulation of the copper importer CTR1 and show aggregation of ATP7A in distinct focal points near engulfed spores (Figures 3A and 3B). However, quantification of copper ions from infected and non-infected macrophages showed no significant difference in the isolate host cell fractions (Figure 3E), reflecting the situation in M. tuberculosis in which there was no significant difference in macrophage copper concentration between extracellular and intracellular bacteria despite an observed upregulation of host CTR1 (Wagner et al., 2005). However, addition of the permeable copper chelator DTTM increased spore survival of A. fumigatus spores when encountering macrophages (Figure 3G) similar to the situation in C. albicans. These data suggest that there might be a difference in biodistribution of copper in whole animals and isolated macrophages in vitro and, together, support a conserved host copper transport response to microbes in general.

Efforts to genetically assess the role of ATP7A, however, are difficult. Specific mutations in this protein can cause Menkes disease in humans (Woinant and Trocello, 2014), and whereas patients suffering from this hypocupric condition have been reported to suffer from reoccurring urinary tract infections (Tümer and Möller, 2010; Wheeler and Roberts, 1976), ATP7A gene deletion animal models are extremely sick, thus in effect precluding their use in infection studies (Madsen et al., 2008; Mercer, 1998). As morpholino technology allows for manageable assessment of nearly lethal mutations in zebrafish, we used this technology to further query a role for this protein in IA, specifically by testing the hypothesis that the reduced colonization the ΔaceA mutant would be restored to wild-type levels in the ATP7A morphant line. Although we found significantly increased growth of wild-type A. fumigatus in this zebrafish mutant, this was not the case for ΔaceA (Figure 3F).

Although acknowledging that morpholino experimentation has limitations, these results did nevertheless suggest that other host mechanisms were involved and spurred our interest in asking whether phagocyte NADPH oxidase (PHOX) activity could also contribute to host dampening of ΔaceA invasion. Phagocytes generate ROI upon infection through activity of the PHOX complex, known as the initial respiratory burst (Hogan and Wheeler, 2014). The complex catalyzes the production of O$_2^-$ that is subsequently converted to H$_2$O$_2$ (Panday et al., 2015). If copper is mobilized into this environment, it can potentiate the redox potential and can thereby form highly reactive DNA-damaging ’OH via Fenton chemistry (Benov, 2001). Mutations in PHOX are associated with a human disease, CGD, an indicator of susceptibility to IA (Pollock et al., 1995). Using both zebrafish and murine CGD models, we found ΔaceA survival restored to wild-type levels upon inactivation of the PHOX complex (Figure 4). Additionally, biochemical inhibition of host PHOX by DPI support an important role for PHOX in contributing to the phenotype observed in the ΔaceA mutant (Figure 4). Although contribution of ROI detoxification mechanisms on virulence of the two pathogens C. neoformans and C. albicans has been reported (Cox et al., 2003; Frohner et al., 2009; Gleason et al., 2014; Martchenko et al., 2004; Narasipura et al., 2003, 2005; Xu et al., 2013), a direct connection to the copper-regulon was not examined in these species. It appears, at least in the host/ A. fumigatus interaction, that host ROI and copper responses cannot be clearly separated (Figure 6).

Dual Nature of Aspergillus fumigatus Countermeasures: Copper Efflux and ROI Defense

Until now, regulation of copper homeostasis in eukaryotic human pathogens has been only explored in two fungi, C. albicans and...
C. neoformans. In C. albicans, a homolog of Cup1p only detoxifies residual copper when the copper-exporting ATPase Crp1 is compromised (Weissman et al., 2000). Deletions of both crp1 and ctr1 resulted in reduced virulence of infected mice (Mackie et al., 2016). In C. neoformans, the metallothioneins Cmt1/2 are important for copper detoxification in the lung (Ding et al., 2013), whereas the copper importers Ctr1/4 play a major role during infection of the brain, suggesting a tissue-specific host strategy to combat pathogens (Sun et al., 2014). Our studies define yet another tactic taken by the filamentous fungus A. fumigatus in defending from host copper defenses that involves not only copper-binding transcription factor regulation of a copper ATPase transporter but also, critically, regulation of ROI defenses (Figure 6).

Experimentation supported this hypothesis on both fronts. Constitutive expression of either crpA or the transcription factor atfA, shown to govern spore ROI detoxification mechanisms (Hagiwara et al., 2009, 2014, 2016; Pereira Silva et al., 2017), rescued survival of the ΔaceA mutant in confrontations with macrophages (Figure 5) and supports the view that copper mobilized by host cells partially exerts its lethality by potentiating host ROI toxicity. In S. cerevisiae, similar transcriptional control of SOD1 by Ace1p was observed (Gralla et al., 1991). Thus, we show that, in contrast to the copper-defense tactics of C. neoformans (metallothionein) and C. albicans (both metallothionein and transporter), AceA regulation of the ATPase CrpA and ROI defense mechanisms are the primary host countermeasures in A. fumigatus (Figure 6).

The fact that activation of either mechanism (e.g., CrpA-mediated transport or AtfA ROI activation) were sufficient to rescue ΔaceA survival blurs the line between which fungal mechanism is most important and—similar to the intertwined contributions of copper transport and PHOX systems in host response above—reinforces the interconnectedness of both fungal responses to copper extremes. Recent studies in C. albicans show a distinct response of ROI defense mechanism toward different copper environments during infection (Broxton and Culotta, 2016; Li et al., 2015), suggesting that a similar connection as demonstrated in A. fumigatus in this study could represent a common maneuver in other fungal pathogens.

Considering that P-type ATPase proteins are considered therapeutic targets due to their accessibility on cell membranes, coupled with the recent progress in specifically targeting a microbial P-type ATPase (Kirk, 2015; Nova-Cueto et al., 2014; Turner, 2016), efforts to target CrpA may hold promise for future work.

**EXPERIMENTAL PROCEDURES**

**Fungal Strains and Culture Conditions**

A. fumigatus strains used in this study are listed in Table S1. Strains were grown on solid glucose minimal medium without copper (GMM) at 37°C with appropriate supplements (Shimizu and Keller, 2001). For pyrG auxotrophs, the growth medium was supplemented with 5 mM uridine and uracil. Conidia were harvested in 0.01% Tween 80 and enumerated using a hemocytometer. For RNA analysis, all strains were inoculated into 50 mL of liquid GMM minus copper at 5 × 10⁵ conidia/mL in duplicate and grown at 37°C and 250 rpm for 24 hr in ambient light conditions. Copper was added for 1 hr at a final concentration of 200 μM. For growth assays all strains indicated number of conidia were inoculated in 2 μL on solidified (Noble Agar; Difco; BD) GMM containing indicated supplements, respectively, and incubated for 2–4 days as indicated at 37°C in the dark. For spore quantification, 1 × 10⁸ were mixed with 10 mL of hand-warm GMM containing agar and the indicated copper concentration and plated on 10 mL of the same solidified media in Petri dishes. For harvesting spores for macrophage survival assays, all strains were grown for 3 days at 37°C in the dark on GMM plus 1 μM copper to ensure comparable growth and melanization of spores. For CFU enumeration, spores were plated on GMM plus 1 μM copper and incubated for 2 days at 37°C in the dark. For zebrafish larvae infection, fungal strains were inoculated onto GMM plates at a concentration of 1 × 10⁶ conidia per plate using an overlay method and grown for 3 days at 37°C.

**Fungal Transformation and Deletion Constructs**

Deletion fragments were created by double-joint fusion PCR, and transformation was carried out as previously described (Palmer et al., 2008); (d’Enfert, 1996) using primers listed in Table S2. DNA of transformants was isolated as described by Green and Sambrook (2012). Integration of the transformation construct was confirmed by diagnostic PCR using primer pairs as indicated in Figures S6–S9. Single integration was confirmed by Southern analysis as described by Green and Sambrook (2012) (Figures S6–S9).

**Gene Expression Analysis**

Mycelia were harvested by filtration through Miracloth (Calbiochem). Total RNA was extracted with TRIzol reagent (Invitrogen), following the manufacturer’s protocol. Northern analysis was performed as described by Green and Sambrook (2012). Probes for northern analysis were constructed at regions internal to the gene of interest using primers listed in Table S2 (“gene”-F/“gene”-R) and labeled with dCTP α²⁵P.

**Protein Biochemistry and Histochemistry**

Infected and non-infected BMDMs (see below) were lysed with 0.5% SDS on ice for 5 min before an equal volume of PBS was added. Protein concentration was quantified using an Epoch2 microplate reader (BioTek) and equal amounts were reconstituted in 2× loading dye. Western blotting was performed according to standard procedures (Green and Sambrook, 2012). For fluorescent detection of ATP7A, infected and non-infected bone marrow-derived macrophages were cultivated as described below, but on microscopy glass coverslips on the bottom of the wells. Cells were incubated with ATP7A and a fluorescently labeled secondary antibody. Coverslips were mounted onto a pre-cleaned microscope slide. Images were taken with a Zeiss AxioMager A10.

**Phylogeny and Data Analysis**

For phylogenetic analysis, reviewed and curated sequences of interest from the Swiss-Prot database (http://www.uniprot.org) of proteins were retrieved and aligned together with A. fumigatus protein sequences (www.aspergillus.org) (Cerqueira et al., 2014) using MAFFT (http://mafft.cbrc.jp/alignment/software/) (Katoh et al., 2002) and http://www.microbesonline.org/fasttree/ (Price et al., 2009).

**Copper Quantification**

Quantification of copper was carried out after spores were challenged with activated murine bone marrow macrophages for 2 hr. Cells were permeabilized with 0.5% SDS as described below. Spores were separated from cell lysate by centrifugation. Cell lysates were sterile filtered before analysis. Remaining spore pellets were reconstituted in 500 μL of deionized water and enumerated using a hemocytometer. Equal amount of spores were sonicated for 30 min before analysis. An Agilent 8800 ICP-MS was used to quantify copper in the samples after an acid digestion with nitric acid and further dilution with doubly deionized water. Sc was used as internal standard at 10 ng/mL to quantify by the external calibration method with reagent blank correction (less than 0.1 ng/mL) as previously described (Subramanian Vignesh et al., 2013).

**Murine AMΦ Isolation**

Specific pathogen-free C57BL/6J and Swiss ICR mice (8–12 weeks old, equal ratio of females and males) were used in this study purchased from Harlan Laboratories. Bronchoalveolar lavage fluid (BALF) were collected from 12–20
mice, pooled, and seeded at a density of 1 × 105 cells/well, and allowed to rest overnight in a 37°C humidified incubator (5% CO2) prior to use.

**Murine Bone Marrow Macrophage Differentiation and Activation**
Bone marrow was obtained by aseptically flushing the femurs and tibias of 8- to 10-week-old C57BL/6J mice (equal ratio of females and males). Cells were incubated for 7 days in a 37°C humidified incubator (5% CO2) with media replacement and removal of non-adherent cells performed every 2–3 days before use.

**Murine Alveolar and Bone-Marrow-Derived Macrophage Killing Assays**
For metal quantification (1 × 105 cells/well) and killing assays (1 × 105 cells/well), spores were incubated with cells in a 3:1 (spore:cell ratio) plus indicated supplements in complete AM medium. Cells and spores were centrifuged at 300 × g for 5 min before incubation for 1 hr at 37°C in a cell incubator. After 1 hr, the media were aspirated, and non-adherent spores were then washed away with PBS before fresh media plus indicated supplements were added to the cells and incubated for 1 hr at 37°C in a cell incubator. Cells were washed and lysed, and spores were enumerated. From each well, spores were plated in three 1:1 serial dilutions in 200 µL in duplicate, starting with 500 spores per plate as the highest amount of spores. The initial spore solution in complete macrophage media was enumerated and plated in a similar fashion starting with 100 spores per plate in duplicate.

**Murine Infection Model**
Six-week-old ICR female mice were used in this assay. In the non-neutropenic (cortisone acetate) model, mice were injected subcutaneously with cortisone acetate (300 mg/kg) 3 days prior to infection, on the day of their infection, 3, 7, and 11 days post-infection. In the neutropenic (cyclophosphamide) model, mice were injected subcutaneously with cyclophosphamide (150 mg/kg) and cortisone acetate (150 mg/kg) 3 days prior to infection, and with cyclophosphamide (150 mg/kg) on the day of their infection, 3 and 6 days post-infection. The mice were infected intranasally with 5 × 105 dormant conidia. Mortality was monitored for 21 days. For histopathology, mice were sacrificed 2 days after infection, and their lungs were removed staining with Grocott’s methenamine silver stain (GMS) (fungal staining) and H&E (tissue and nuclear staining). For fungal burden, infected mice were sacrificed on the second day post-infection, and their lungs were removed staining with Grocott’s methenamine silver stain (GMS) (fungal staining), and H&E (tissue and nuclear staining). For fungal burden, infected mice were sacrificed on the second day post-infection, and their lungs were removed staining with Grocott’s methenamine silver stain (GMS) (fungal staining), and H&E (tissue and nuclear staining). For fungal burden, infected mice were sacrificed on the second day post-infection, and their lungs were removed staining with Grocott’s methenamine silver stain (GMS) (fungal staining), and H&E (tissue and nuclear staining). For fungal burden, infected mice were sacrificed on the second day post-infection, and their lungs were removed staining with Grocott’s methenamine silver stain (GMS) (fungal staining), and H&E (tissue and nuclear staining).

**CqD Infection Model**
C57BL/6J mice were purchased from The Jackson Laboratory. Mice with an inactivation of X-linked Cybb (X-CqD mice) in the C57BL/6J (backcrossed >15 generations) and wild-type littermates controls were obtained from in-house colonies (Pollock et al., 1995). Mice were used between 10 and 21 weeks of age. Mice received 30,000 conidia via nasopharyngeal instillation. Mice were sacrificed after 24 hr, and lungs were then homogenized and plated for CFU on GMM for 2 days at 37°C. To quantitate total fungal DNA, homogenized lungs were further bead beaten with acid-washed glass beads, and DNA was extracted with the DNeasy Blood & Tissue Kit (QIAGEN; 69504). All DNA quantity and quality were assessed with BioTek Gen5 microplate reader (BioTek Instruments) previously described (Li et al., 2011).

**Zebrafish Care and Maintenance**
Adult zebrafish were housed on a system with regulated water temperature at 28.5°C, pH, and conductivity in a room programmed with a light/dark cycle of 14 and 10 hr, respectively, and fed twice daily.

**Zebrafish Infection Model**
All zebrafish infection experiments were performed as described (Knox et al., 2014). Morpholino-mediated genetic knockdown of p22phox or atp7a was obtained as previously described (Tazulin et al., 2014). Immediately following microinjection, 8–12 randomly selected larvae from each condition were individually homogenized and spread evenly on GMM agar plates containing 1 µM copper for time zero CFU enumeration. Similarly, at 24 hr post-infection (hpi), 8–12 larvae were randomly selected and processed in a similar manner.

**Statistical Analyses**
Statistical differences of data were analyzed using the GraphPad Prism 5 software package (GraphPad Software). For fungal CFU-forming experiments from macrophages, spore counting from fungal growth plates, diameter measurements in H2O2 stress tests, and copper quantification, p values were calculated with one-way ANOVA for multiple comparisons and adjusted with Bonferroni’s or Holm-Sidak correction and non-paired Student’s t test where two groups were compared. All error bars given represent SDs. For larval zebrafish CFU experiments, data from four independent replicates were pooled, and significance was determined with ANOVA with results summarized using least-squares adjusted means and SEs.

**Ethics Statement**
All animal experiments were carried out in strict accordance to the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health. Experiments were performed with the approval of the Institutional Animal Care and Use Committee (IACUC) of Tel Aviv University, the University of Wisconsin-Madison, and Washington University in St. Louis, respectively. All efforts were made to minimize the number of animals used and animal suffering.

**SUPPLEMENTAL INFORMATION**
Supplemental Information includes Supplemental Experimental Procedures, nine figures, and two tables and can be found with this article online at dx.doi.org/10.1016/j.celrep.2017.04.019.

**AUTHOR CONTRIBUTIONS**

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Candida albicans


Aspergillus

Cryptococcus neoformans

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Supplemental Information

*Aspergillus fumigatus* Copper Export Machinery and Reactive Oxygen Intermediate Defense Counter Host Copper-Mediated Oxidative Antimicrobial Offense

Philipp Wiemann, Adi Perevitsky, Fang Yun Lim, Yana Shadkchan, Benjamin P. Knox, Julio A. Landero Figueora, Tsokyi Choera, Mengyao Niu, Andrew J. Steinberger, Marcel Wüthrich, Rachel A. Idol, Bruce S. Klein, Mary C. Dinauer, Anna Huttenlocher, Nir Osherov, and Nancy P. Keller
Supplemental Experimental Procedures

Fungal strains and culture conditions

*Aspergillus fumigatus* strains used in this study are listed in Table S1. Strains were maintained as glycerol stocks and activated on solid glucose minimal medium (GMM) at 37 °C with appropriate supplements (Shimizu and Keller, 2001). The trace elements for all experiments were used from a 1000x stock containing 22 g/L ZnSO₄ x 7 H₂O, 11 g/L H₂BO₃, 5 g/L MnCl₂ x 4 H₂O, 1.6 g/L FeSO₄ x 7 H₂O, 1.6 g/L CoCl₂ x 5 H₂O, and 1.1 g/L (NH₄)₃MoO₄ x 4H₂O. To adjust for different Cu conditions, Cu was added from a 100 mM CuSO₄ stock solution. The pH of all media was adjusted to 6.0 to prevent Cu precipitation. For experiments in Fig. S2D, trace elements were prepared as described above but with additional 10 g/L EDTA. For solidified media, Noble Agar (Difco™, BD, USA) was used. For pyrG auxotrophs, the growth medium was supplemented with 5 mM uridine and uracil. Conidia were harvested in 0.01% Tween 80 and enumerated using a hemocytometer. For RNA analysis all strains were inoculated into 50 mL of liquid GMM -Cu at 5 x 10⁶ conidia/mL in duplicate and grown at 37°C and 250 rpm for 24 h in ambient light conditions. Cu was added for 1 h at a final concentration of 200 µM. The mycelium was harvested and lyophilized before RNA extraction. For growth assays all strains indicated number of conidia were inoculated in 2 µL on solidified (Noble Agar, Difco™, BD, USA) GMM containing indicated supplements, respectively, and incubated for 2-4 days as indicated at 37 °C in the dark. For spore quantification, 1 x 10⁸ were mixed with 10 mL hand warm GMM containing agar and the indicated Cu concentration and plated on 10 mL of the same solidified media in petri dishes. Spores were counted in triplicates from 1 cm² disks punched out of the plates after 5 days of incubation at 37 °C in the dark. For harvesting spores, cultures were harvested in 0.01% Tween 80 and brought to a volume of 50 mL in a 50 mL screw-cap tube. Following centrifugation at 900 g for 15 minutes the conidia pellet was resuspended in 50 mL PBS, pelleted a second time, and resuspended in a final volume of ~3 mL PBS before being enumerated with a hemocytometer and adjusted to a final concentration of 1.5 x 10⁸ conidia/mL. Conidia stocks used for zebrafish larvae infection experiments were used within 4 days of preparation.

Fungal transformation and deletion constructs

Deletion fragments were created by double-joint fusion PCR. Briefly, ~1-kb fragments flanking the targeted deletion region were amplified by PCR from *Aspergillus fumigatus* strain CEA17 genomic DNA using the primer pairs (gene)-5F/[gene]-5R and [gene]-3F/[gene]-3R, respectively (where F indicates forward, and R indicates reverse) (Table S2). The *Aspergillus parasiticus* pyrG marker gene was amplified from the plasmid pJW24 (Calvo et al., 2004) using the primer pair pyrG_promF/pyrG_term_R (Table S2). The primers (gene)-5R and [gene]-3F contain complement sequences to the primers pyrG_promF and pyrG_term_R at their 5'-region, respectively (Table S2). The fusion construct was created by PCR containing 5' and 3' gene flanks and the pyrG gene fragment functioning as templates and primers simultaneously. The final PCR fusion product was amplified using primer pairs (gene)-5F/[gene]-3R and the previously PCR-generated fusion construct as template. For over-expression of crpA the hygromycin resistance cassette was amplified from pUCH2-8 (Alexander et al., 1998) using the primer pair PUCH28-F/-R and fused to the *A. nidulans* gpdA promoter (Yin et al., 2012) using primer pairs gpdA-F/-R. The crpA open reading frame and the 5′ region were amplified with primer pairs OEcrpA-F/-R and OEcrp5-F/-R, respectively. The two *A. fumigatus* fragments were fused to the hygromycin/gpdA hybrid construct using primer pair OEcrp5-F/OEcrpA-R. Transformation was performed as previously described (Palmer et al., 2008). For selection of *ΔaceA* transformants, Cu and uracil/uridine were omitted in the selection media. For selection of *ΔmacA* transformants, uracil/uridine were omitted in the selection media and Cu was supplemented to 10 µM final concentration. For selection of OE::crpA strains, GMM media containing 10 µM Cu was supplemented with 120 µg/mL hygromycin. For creating the *ΔaceA/OE::atf1* we first created a *ΔargB* strain from CEA17 KU80 as described for the other gene deletions above. Transformants were selected on media containing 1 g/L arginine. Subsequently we transformed *ΔargB* with a construct that had the two pyrG flanks fused to each other using the primer pair XX. Proteolipids were selected on media containing 5 mM uracil/uridine, 1 g/L arginine and 1 g/L 5-fluoro-orotic acid (5-FOA) as previously described (d’Enfert, 1996) creating strain ΔargG/ΔargB. This strain was used to delete *ΔaceA* as described above creating strain ΔargB/ΔaceA. For overexpression of atf1, a gpdA promoter/argB/5′ atf4 fusion construct was created using primer pairs 60/61 and 62/63 and argBF (5′-agtctctatgtaagatgggagt/gpdR (5′-catgggtgtcgtcagctg)) (Table S2). PJMP10 (Yin et al., 2012) was
used as template DNA to amplify the initial gpdA promoter/argB construct and fused to constructs amplifying the 5' region non-coding and open reading frame of atf1. For DNA isolation, A. fumigatus strains were grown for 24 h at 37°C in steady state liquid GMM, supplemented with appropriate Cu concentrations. DNA isolation was performed as described by (Green and Sambrook, 2012). For multiplex diagnostic PCR, primer pair 'gene'-F/'gene'-R were used to identify transformants that lost the respective gene locus and primer pair gpd_int-F/gpd_int-R as internal control (Table S2). Integration of the transformation construct was confirmed by diagnostic PCR using primer pairs as indicated in Fig. S12-20 and Table S2 (data not shown). Single integration was confirmed by Southern analysis as described by (Green and Sambrook, 2012) using P32-labelled probes created by amplification of the respective construct using primer pairs indicated in Fig. S12-20 and Table S2.

Gene expression analysis
Mycelia were harvested by filtration through Miracloth (Calbiochem). Total RNA was extracted with TRIzol reagent (Invitrogen) from freeze-dried mycelia, following the manufacturer’s protocol. Northern analysis was performed as described by (Green and Sambrook, 2012). Probes for northern analysis were constructed at regions internal to the gene of interest using primers listed in Table S1 ('gene'-F/'gene'-R) and labeled with dCTP αP32.

Protein bio- and histochemistry
Infected and non-infected bone marrow derived macrophages (see below) were lysed with 0.5 % SDS on ice for 5 min before an equal volume of PBS was added. Protein concentration was quantified using an Epoch2 microplate reader (BioTek) and equal amounts were reconstituted in 2x LDS buffer [0.5 M Tris/HC1 (pH 8.5), 20% glycerol, 4% LDS, 1 mM EDTA, 0.44 mM Coomassie, 5% β-mercaptoethanol] and stored at −20°C until ready for use. For gel electrophoresis, the samples were heated at 95°C for 5 min prior to loading and resolved using a 10% Bis Tris gel in MOPS running buffer and transferred onto a PVDF membrane (Millipore) at 15 V for an hour on a Trans-Blot® SD Semi-Dry transfer cell (Bio-Rad) using standard manufacturer’s protocol. The membranes were then visualized using Ponceau red staining for successful transfer and blocked for 2 h at room temperature in TBS-T containing 5% non-fat milk. The membranes were then incubated in 1:1000 (v/v) primary rabbit anti mouse Ctrl antibody (Invitrogen) or 1:1000 (v/v) primary horseradish peroxidase (HRP) conjugated mouse GPDH antibody (Invitrogen) overnight at 4°C in TBS-T containing 0.1% non-fat milk and washed four times for 15 min each in TBS-T. For Ctrl detection, the membranes were then incubated in 1:5000 (v/v) secondary HRP-conjugated goat anti rabbit antibody (Thermo Fisher) in TBS-T containing 0.1% non-fat milk for 1 h at room temperature and washed in TBS-T as described above. Samples were then incubated in ClarityTM Western ECL substrate (Bio-Rad) for 5 min following manufacturer’s protocol and subjected to 2 min film exposure. Membranes were stripped in 1% Tween20, 0.1% SDS, pH2.2 for 15 min at 70°C.

For fluorescent detection of ATP7A, infected and non-infected bone marrow derived macrophages were cultivated as described below, but on microscopy glass coverslips on the bottom of the wells. Cells were washed twice with 1 ml of ice-cold PBS, and then fixed for 10 min at 25°C using 4% paraformaldehyde. The cells were then permeabilized with 0.1% Triton X-100 in PBS for 5 min, blocked for blocked for 2 h at room temperature in TBS-T containing 5% non-fat milk, and then probed with the goat anti mouse ATP7A antibodies (1:500) (Invitrogen) overnight at 4°C in TBS-T containing 0.1% non-fat milk and washed four times for 15 min each in TBS-T. For ATP7A detection, the membranes were then incubated in 1:5000 (v/v) secondary HRP-conjugated goat anti rabbit antibody (Thermo Fisher) in TBS-T containing 0.1% non-fat milk for 1 h at room temperature and washed in TBS-T as described above. Samples were then incubated on a Cite® light source (BioTek) and equal amounts were reconstituted in 2x LDS buffer [0.5 M Tris/HC1 (pH 8.5), 20% glycerol, 4% LDS, 1 mM EDTA, 0.44 mM Coomassie, 5% β-mercaptoethanol] and stored at −20°C until ready for use. For gel electrophoresis, the samples were heated at 95°C for 5 min prior to loading and resolved using a 10% Bis Tris gel in MOPS running buffer and transferred onto a PVDF membrane (Millipore) at 15 V for an hour on a Trans-Blot® SD Semi-Dry transfer cell (Bio-Rad) using standard manufacturer’s protocol. The membranes were then visualized using Ponceau red staining for successful transfer and blocked for 2 h at room temperature in TBS-T containing 5% non-fat milk. The membranes were then incubated in 1:1000 (v/v) primary rabbit anti mouse Ctrl antibody (Invitrogen) or 1:1000 (v/v) primary horseradish peroxidase (HRP) conjugated mouse GPDH antibody (Invitrogen) overnight at 4°C in TBS-T containing 0.1% non-fat milk and washed four times for 15 min each in TBS-T. For Ctrl detection, the membranes were then incubated in 1:5000 (v/v) secondary HRP-conjugated goat anti rabbit antibody (Thermo Fisher) in TBS-T containing 0.1% non-fat milk for 1 h at room temperature and washed in TBS-T as described above. Samples were then incubated in ClarityTM Western ECL substrate (Bio-Rad) for 5 min following manufacturer’s protocol and subjected to 2 min film exposure. Membranes were stripped in 1% Tween20, 0.1% SDS, pH2.2 for 15 min at 70°C.

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Phylogeny and data analysis
For phylogenetic analysis, reviewed and curated sequences from the Swiss-Prot database (www.uniprot.org) of proteins containing a Cu-fist domain were retrieved and aligned together with the protein sequences of Afu6g07780/AceA, Afu1g13190/MacA, and Afu2g01190/CufA (www.aspergillus.org) (Cerqueira et al., 2014) using MAFFT (http://mafft.cbrc.jp/alignment/software/) (Katoh et al., 2002) and (http://www.microbesonline.org/fasttree/) (Price et al., 2009). Results were visualized using FigTree (http://tree.bio.ed.ac.uk/software/figtree/). For alignment of HMD ATPases, reviewed and curated sequences from the Swiss-Prot database (www.uniprot.org) were retrieved and aligned with homologous sequences from fungal pathogens as described above for Cu-fist domain proteins. For visualization of protein alignments, ClustalW (MegAlign, DNASTar, Madison, WI, USA) was used.
The plates were incubated for 24 h, and the numbers of colony forming units (CFU) were counted. TNF
hematoxylin and eosin (H&E; tissue and nuclear staining). For fungal burden, infected mice were sacrificed on the
were removed and sent for histological staining wit
Mortality was monitored for 21 days. For histopathology, mice were sacrificed two days after infection and their lungs
intranasally with 5 x 10
Six week old ICR female mice were used in this assay.

Murine infection model
Survival of spores was calculated by counting colony forming units in comparison to the input control.

duplicate, starting with 500 spores per plate as the highest amount of spores. The initial spore solution in complete
PBS. Cells were lysed with 0.5 % SDS on ice for 5 min before an equal volume of PBS was added. Spores were
adherent spores were then washed away with PBS before fresh media plus indicated supplements was added to
the cells and incubated for 1 h at 37 °C in a cell incubator. After 1 h the media was aspirated and non-adherent cells were then washed away with PBS before fresh media plus indicated supplements was added to
the cells and incubated for 1 h at 37 °C in a cell incubator. Media was aspirated and cells were washed twice with
PBS. Cells were lysed with 0.5 % SDS on ice for 5 min before an equal volume of PBS was added. Spores were
 enumerated using a hemocytometer. Equa

Copper quantification
Quantification of Cu was carried out after spores were challenged with activated murine bone marrow macrophages
for 2 hours. Cells were permeabilized with 0.5% SDS as described below. Spores were separated from cell lysate by
centrifugation. Cell lysates were sterile filtered before analysis. Remaining spore pellets were reconstituted in 500 µL
deionized water and enumerated using a hemocytometer. Equal amount of spores were sonicated for 30 min before
analysis. An Agilent 8800 ICP-MS was used to quantify Cu in the samples after an acid digestion with nitric acid and
further dilution with doubly deionized water. Sc was used as internal standard at 10 ng/ml to quantify by the external
calibration method with reagent blank correction (less than 0.1 ng/ml) as previously described (Subramaniam Vignesh
et al., 2013).

Murine Alveolar Macrophage Isolation
Specific pathogen-free C57BL/6J and Swiss ICR mice were used in this study, purchased from Harlan Laboratories
Inc. Mice were housed and cared for in compliance with guidelines of the University of Wisconsin Animal
Care.Murine alveolar macrophages (AMΦ) were obtained from bronchoalveolar lavage fluid (BALF) of C57BL/6J
mice. Briefly, mice were euthanized and trachea were prepared in situ. BALF were collected by 6-8 sequential flushing
of the lung with 1 mL of sterile Dulbecco’s PBS without calcium and magnesium (dPBS – Ca – Mg) containing 1
mM EDTA using a 20-22 gauge catheter. Depending on the number of AMΦ needed per experiment, BALF of 12-20
mice were pooled and centrifuged for 10 mins at 600xg. The pellet was resuspended in DMEM (Corning Cellgro: 10-
013-CV) supplemented with 10% heat-inactivated FBS (Atlanta Biologicals; S11150) and 1% penicillin/streptomycin
(Life Technologies; 10378016). Cells were seeded at a density of 1x10² cells/well and allowed to rest overnight in a
37°C humidified incubator (5% CO₂) prior to use.

Murine Bone Marrow Macrophage Differentiation and Activation
Bone marrow was obtained by aseptically flushing the femurs and tibias of 8-10 week old C57BL/6J mice with 2-3
mL of sterile, ice cold dPBS – Ca – Mg. Upon red blood cell lysis, bone marrow was cultured in DMEM supplemented
with 20% L929-conditioned medium, 10% heat-inactivated FBS, and 1% pen/strep for seven days in a 37°C humidified incubator (5% CO₂) with media replacement and removal of non-adherent cells performed every 2-3 days. Differentiated bone-marrow derived macrophages (BMDMΦ) were maintained in DMEM supplemented with 10% heat-inactivated FBS and 1% pen/strep and activated with either 10 ng/mL murine recombinant GM-CSF (Peprotech; 315-03) or murine recombinant IFN-γ (Peprotech; 315-05) + LPS from E. coli 055:B5 (Sigma-Aldrich; L6529) for 18 hours prior to use. L929-conditioned medium was prepared by culturing L929 fibroblast cells in DMEM supplemented with 10% heat-inactivated FBS, 1% GlutaMAX™ (Life Technologies; 35050), and 1% pen/strep for
10 days in a 37°C humidified incubator (5% CO₂). The L929-conditioned medium was sterile-filtered through a 0.22
µM filter and stored at -80°C until ready for use.

Murine alveolar and bone marrow derived macrophage killing assays
For metal quantification (1 x 10⁷ cells/well) and killing assays (1 x 10⁵ cells/well) spores were incubated with cells in a
3:1 (spore:cell ratio) plus indicated supplements in complete alveolar macrophage media. Cells and spores were centrifuged at 300 g for 5 min before incubation for 1 h at 37 °C in a cell incubator. After 1 h the media was aspirated and non-adherent spores were then washed away with PBS before fresh media plus indicated supplements was added to
the cells and incubated for 1 h at 37 °C in a cell incubator. Media was aspirated and cells were washed twice with
PBS. Cells were lysed with 0.5 % SDS on ice for 5 min before an equal volume of PBS was added. Spores were
enumerated using a hemocytometer. From each well, spores were plated in three 1:1 serial dilutions in 200 µL in
duplicate, starting with 500 spores per plate as the highest amount of spores. The initial spore solution in complete
macrophage media was enumerated and plated in a similar fashion starting with 100 spores per plate in duplicate.
Survival of spores was calculated by counting colony forming units in comparison to the input control.

Murine infection model
Six week old ICR female mice were used in this assay. Mice were injected subcutaneously with cortisone acetate (300
mg/kg) 3 days prior to infection, on the day of their infection, 3, 7 and 11 days post infection. The mice were infected
intranasally with 5 x 10⁷ dormant conidia, suspended in 20 µL of PBS + 0.02% Tween 20 (10 µL in each nostril).
Mortality was monitored for 21 days. For histopathology, mice were sacrificed two days after infection and their lungs
were removed and sent for histological staining with Grocott's methenamine silver stain (GMS; fungal staining) and
hematoxylin and eosin (H&E; tissue and nuclear staining). For fungal burden, infected mice were sacrificed on the
second day post infection, their lungs were removed and homogenized, and the homogenates were plated on YAG.
The plates were incubated for 24 h, and the numbers of colony forming units (CFU) were counted. TNF-α levels were
measured two days post infection by ELISA of the supernatant from whole lung homogenates. ELISA was performed according to the instructions in the Peprotech kit (Murine TNF-α Mini ABTS ELISA Development Kit, Peprotech Worldwide).

CGD infection model
C57Bl/6J mice were purchased from The Jackson Laboratory. Mice with an inactivation of X-linked Cybb (X-CGD mice) in the C57Bl/6J (backcrossed >15 generations) and WT littermate controls were obtained from in-house colonies (Pollock et al., 1995). Mice were maintained in specific pathogen-free conditions and used between 10-21 weeks of age. All experiments were conducted as approved by the Washington University in St. Louis Animal Studies Committee. A. fumigatus strains were grown as described above. Conidia were harvested by pipetting 5-10 mL of PBS onto each plate, gently scraping with a cell scraper, filtered through Miracloth and a 40 μM strainer and dilutions counted on a hemacytometer. Mice received 30,000 conidia suspended in 25 μL PBS via nasopharyngeal installation. Mice were anesthetized with a ketamine/dexdomitor cocktail prior to A. fumigatus instillation and anesthesia was reversed by atipamezole hydrochloride approximately 2 minutes after instillation. Total time under anesthesia ranges from 5-7 minutes per mouse. To determine fungal burden, mice were sacrificed after 24 h and lungs were then homogenized in 1mL PBS using a bead homogenizer and dilutions plated for CFU on GMM for 2 days at 37°C. To quantitate total fungal DNA, homogenized lungs were further bead beaten with acid washed glass beads and DNA was extracted with the DNeasy Blood & Tissue Kit (Qiagen- 69504). All DNA quantity and quality were assessed with BioTek Gen5 microplate reader (BioTek Instruments, Inc.). Quantitative PCR was modified from previously described (Li et al., 2011). A TaqMan probe/primer set [AF 18S for: 5′ GGCCTTAATAGCCCGGT-3′ AF 18S rev: 5′-TGAGCGGATAGTCCCCCTAA-3′ AF 18S probe: 5′-FAM-AGCCAGGCCCCGCAAATG- TAMRA-3′ (Integrated DNA Technologies)] was used to amplify the 18S rRNA gene of A. fumigatus (GenBank accession no. AB008401). A six point standard curves were calculated using serially diluted genomic DNA of both CEA10 and ΔaceA strain. Total fungal DNA from total DNA extracted was calculated utilizing the appropriate standard curve and normalizing to input DNA concentration. DNA samples were analyzed in triplicate by using C1000 Touch thermal cycler (Bio-Rad). Data analyses were conducted using GraphPad Prism 6 (GraphPad Software, Inc.).

Zebrafish care and maintenance
Adult zebrafish were housed on a system with regulated water temperature, pH, and conductivity in a room programmed with a light/dark cycle of 14 hours and 10 hours, respectively, and fed twice daily. Larval zebrafish were kept at 28.5 °C in E3 buffer. During infection experiments, methylene blue was omitted from E3 buffer.

Larval zebrafish infection model
All larval zebrafish infection experiments were performed as described (Knox et al., 2014) with few modifications. Morpholino-mediated genetic knockdown of p22phox or atp7a was obtained as previously described (Taufzin et al., 2014) by injecting 3 nL of morpholino into yolks of freshly spawned eggs during the 1-2 cell stage. For infections, manually dechorionated larvae were anesthetized with media supplemented with 0.2 mg/mL Tricaine (ethyl 3-aminobenzoate; Sigma-Aldrich) prior to microinjection at 48 hours post fertilization with 3 nL conidial suspensions (see above) diluted to 1 x 10⁵ conidia/mL with sterile 1% phenol red into the hindbrain ventricle. Immediately following microinjection, 8-12 randomly selected larvae from each condition were individually homogenized and spread evenly on GMM agar plates containing 1 μM Cu for time zero CFU enumeration. Similarly, at 24 hours post infection (hpi) 8-12 larvae were randomly selected and processed in a similar manner. Individual CFU counts from 24 hpi were normalized to the mean from 0 hpi.

Statistical analyses
Statistical differences of data were analyzed using the GraphPad Prism 5 software package (GraphPad Software, Inc, San Diego, CA). For fungal CFU forming experiments from macrophages, spore counting from fungal growth plates, diameter measurements in H₂O₂ stress tests and Cu quantification, p values were calculated with one-way ANOVA for multiple comparisons and adjusted with Bonferroni’s or Holm Sidak correction and non-paired Student’s t test where two groups were compared. For larval zebrafish CFU experiments, data from four independent replicates were pooled and significance determined with analysis of variance with results summarized using least squares adjusted means and standard errors.
Figure S1. Phylogenetic relation and domain architecture of Cu-Fist transcription factors. Related to Table 1.
(A) Phylogenetic analysis of the three *A. fumigatus* Cu-Fist proteins (AceA, MacA and CufA) with characterized fungal Cu homeostasis transcription factors.
(B) Clustal W alignment of Cu-Fist transcription factors. DNA binding motif in red, residues responsible for Cu-binding in Ace-type transcription factors in green and Mac-type transcription factors in blue.
Figure S2. Growth phenotypes of the Cu-Fist mutants on different metal stresses. Related to Figure 1.

(A) 2000 spores of indicated strains grown on solidified glucose minimal medium (GMM) without EDTA with indicated concentration of CuSO₄ for 72 h at 37°C.

(B) 2000 spores of indicated strains grown on solidified GMM with reduced Cu concentrations amended with 100 µM CdSO₄ and FeSO₄, respectively, for 72 h at 37°C.

(C) 2000 spores of indicated strains grown on solidified GMM with indicated Cu concentrations for 48 h at 37°C. BCS = 0 µM Cu with 50 µM bathocuproinedisulfonic acid as Cu chelator.

(D) 2000 spores of indicated strains grown on solidified glucose minimal medium (GMM) with 10 mg/L EDTA with indicated concentration of CuSO₄ for 72 h at 37°C.

(E) 2000 spores of indicated strains grown on solidified GMM with elevated Cu concentrations amended with 500 µM GSH for 72 h at 37°C.

(F) Growth inhibition of spores from indicated strains incubated with 100 µL of 4.5 % (v/v) H₂O₂ according to Thön et al., 2007. Experiment was carried out in triplicate, error bars represent standard deviations and asterisks indicate statistical difference, p < 0.05.
Figure S3: Inflammatory responses, fungal survival and histopathology, and metal quantification of wild-type and ΔaceA infected mice from our infection models. Related to Figure 2, Figure 3, Figure 4 and Figure 5.

(A) Histopathology of infected mice lungs stained with Grocott’s methenamine silver stain (GMS; fungal staining) and hematoxylin and eosin (H&E; tissue and nuclear staining)

(B) Colony forming units (CFU) of wild type and ΔaceA mutant from infected mice lungs. Experiments were carried out in triplicate; error bars represent standard deviations and statistical significance is indicated as $p$. 

value.

(C) TNFα concentrations from infected lungs. Experiments were carried out in triplicate; error bars represent standard deviations and statistical significance is indicated as p value.

(D) Coomassie gel staining, Ponceau membrane staining and Western blot against mouse Ctr1 of *A. fumigatus* grown under Cu deplete and replete conditions. The mouse Ctr1 antibody shows an unspecific cross reaction with *A. fumigatus* proteins of 50 kDa.

(E) Western blot against mouse Ctr1 of murine bone marrow derived macrophages activated with GM-CSF that were unchallenged or challenged with *A. fumigatus* spores for 2h (Fig. 3A). The antibody shows a reaction with a protein of 28 kDa in the *A. fumigatus* infected samples only. The size is distinctively different than the unspecific binding observed against *A. fumigatus* protein extracts shown in panel A.

(F) Total Zn concentration of 1 x 10^7 GM-CSF activated bone marrow derived murine macrophages incubated with 3 x 10^7 spores of the indicated *A. fumigatus* strains for 2h. Experiments were carried out in triplicates; error bars represent standard deviations and statistical significance is indicated by p values.

(G) Total Fe concentration of 1 x 10^7 GM-CSF activated bone marrow derived murine macrophages incubated with 3 x 10^7 spores of the indicated *A. fumigatus* strains for 2h. Experiments were carried out in triplicates; error bars represent standard deviations and statistical significance is indicated by p values.

(H) Total Cu amount from *A. fumigatus* mycelia in Cu replete conditions. The indicated strains were grown for 24 h in GMM –Cu, harvested and freeze dried. Experiments were carried out in triplicates; error bars represent standard deviations and statistical significance is indicated by p values.

(I) Total Cu amount from *A. fumigatus* spores grown collected from solidified media containing 0 µM or 5 µM Cu. Experiments were carried out in triplicates; error bars represent standard deviations and statistical significance is indicated by p values.

(J) Total Cu concentration of 1 x 10^7 GM-CSF activated bone marrow derived murine macrophages incubated with 3 x 10^7 spores of the indicated *A. fumigatus* strains for 2h. Experiments were carried out in triplicates; error bars represent standard deviations and statistical significance is indicated by p values.

(K) Total Cu concentration of 3 x 10^7 spores incubated with 1 x 10^7 GM-CSF activated bone marrow derived murine macrophages for 2h. Experiments were carried out in triplicates; error bars represent standard deviations and statistical significance is indicated by p values.

(L) Colony forming units (CFU) of fungal strains after infection of WT (control) mice and CGD mice after 24h. Two individual experiments are shown with N = 5 mice each; error bars represent standard deviations statistical significance is indicated by p values.
Figure S4: Phylogenetic relation and domain architecture of heavy metal ATPases (HMA) of select species and Cu-dependent expression analysis. Related to Figure 5.

Phylogenetic analysis of A. fumigates HMA with HMA proteins from pathogenic fungi and characterized HMA.

(A) Phylogenetic analysis of A. fumigates HMA with HMA proteins from pathogenic fungi and characterized HMA.

(B) Northern blot analysis of indicated strains grown for 24 h in liquid GMM -Cu at 37°C. To half of the cultures Cu was added to a final concentration of 200 µM for 1 h before harvesting. Indicated genes were hybridized. rRNA visualization as loading as control.

(C) Northern blot analysis of indicated strains grown for 24 h in liquid GMM -Cu at 37°C. To half of the cultures Cu was added to a final concentration of 200 µM for 1 h before harvesting. Indicated genes were hybridized. rRNA visualization as loading as control.
(D) Northern blot analysis of indicated strains grown for 24 h in liquid GMM-Cu at 37°C. To half of the cultures Cu was added to a final concentration of 200 µM for 1 h before harvesting. Indicated genes were hybridized. rRNA visualization as loading as control. The original image was cropped to exclude expression of a strain not relevant for this study.
Fig. S5: Characterization of the metallothionein CmtA in *A. fumigatus* and growth phenotypes of *crpA* and *atfA* over-expression strains on elevated Cu concentrations. Related to Figure 5.

(A) 2000 spores of indicated strains grown on solidified glucose minimal medium (GMM) without EDTA with indicated concentration of CuSO₄ and menadione for 48 h at 37°C.

(B) CFU of spores incubated in GMM supplemented with 5 µM Cu and 10 mM H₂O₂ for 1 h at 37°C. After incubation spores were washed twice and plated in serial dilutions on GMM containing 1 µM Cu. Experiments were carried out in triplicates; error bars represent standard deviations and statistical significance is indicated by p values.

(C) 2000 spores of indicated strains grown on solidified glucose minimal medium (GMM) with indicated concentration of CuSO₄ for 72 h at 37°C.

(D) Colony forming units (CFU) of fungal strains after incubation with murine alveolar macrophages for 2h. Experiments were carried out in triplicates; error bars represent standard deviations and statistical significance is indicated by p values.
(E) 2000 spores of indicated strains grown on solidified glucose minimal medium (GMM) with indicated concentration of CuSO₄ for 72 h at 37°C.

(F) Colony forming units (CFU) of indicated strains from infected mice lungs. N = 10 mice each; error bars represent standard deviations statistical significance is indicated by p values.
Figure S6: Deletion strategy and Southern analysis of ∆aceA, ∆macA and ∆cufA strains. Related to Experimental Procedures.

(A) Deletion strategy for ∆aceA strains. Arrows indicate positions of primers used (Table S2).

(B) Southern blot analysis of recipient strain and ∆aceA strains. Restriction was carried out using the indicated enzymes and probed with the indicated PCR fragments.

(C) Deletion strategy for ∆macA strains. Arrows indicate positions of primers used (Table S2).

(D) Southern blot analysis of recipient strain and ∆macA strains. Restriction was carried out using the indicated enzymes and probed with the indicated PCR fragments.

(E) Deletion strategy for ∆cufA strains. Arrows indicate positions of primers used (Table S2).

(F) Southern blot analysis of recipient strain and ∆cufA strains. Restriction was carried out using the indicated enzymes and probed with the indicated PCR fragments.
enzymes and probed with the indicated PCR fragments.

**Figure S7:** Deletion strategy and Southern analysis of \( \Delta crpA \), OE::crpA and \( \Delta argB \) strains. Related to Experimental Procedures.

(A) Deletion strategy for \( \Delta macA \) and OE::crpA strains. Arrows indicate positions of primers used (Table S2).

(B) Southern blot analysis of recipient strain and \( \Delta crpA \) strains. Restriction was carried out using the indicated enzymes and probed with the indicated PCR fragments.

(C) Southern blot analysis of recipient strain and OE::crpA strains. Restriction was carried out using the indicated enzymes and probed with the indicated PCR fragments.

(D) Deletion strategy for \( \Delta argB \) strains. Arrows indicate positions of primers used (Table S2).

(E) Southern blot analysis of recipient strain and \( \Delta argB \) strains. Restriction was carried out using the indicated enzymes and probed with the indicated PCR fragments.
Figure S8: Deletion strategy and Southern analysis of \(\Delta\Delta\text{argB/pyrG}\) and \(\Delta\Delta\text{argB/aceA}\) strains. Related to Experimental Procedures.

(A) Deletion strategy for \(\Delta\Delta\text{argB/pyrG}\) strains. Arrows indicate positions of primers used (Table S2).

(B) Southern blot analysis of recipient strain and \(\Delta\Delta\text{argB/pyrG}\) strains. Restriction was carried out using the indicated enzymes and probed with the indicated PCR fragments.

(C) Deletion strategy for \(\Delta\Delta\text{argB/aceA}\) strains. Arrows indicate positions of primers used (Table S2).

(D) Southern blot analysis of recipient strain and \(\Delta\Delta\text{argB/aceA}\) strains. Restriction was carried out using the indicated enzymes and probed with the indicated PCR fragments.
Figure S9: Deletion strategy and Southern analysis of ∆atfA OE::atfA and ∆aceA/OE::atfA strains. Related to Experimental Procedures.

A) Deletion strategy for ∆atfA strains. Arrows indicate positions of primers used (Table S2).

B) Southern blot analysis of recipient strain and ∆atfA strains. Restriction was carried out using the indicated enzymes and probed with the indicated PCR fragments.

C) Deletion strategy for OE::crpA strains in indicated backgrounds. Arrows indicate positions of primers used (Table S2).

D) Southern blot analysis of ∆argB strain and OE::atfA strains. Restriction was carried out using the indicated enzymes and probed with the indicated PCR fragments.

E) Southern blot analysis of ∆∆argB/aceA and ∆aceA/OE::atfA strains. Restriction was carried out using the indicated enzymes and probed with the indicated PCR fragments.
Supplemental Tables

Table S1: Fungal strains used in this study. Related to Experimental Procedures.

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