Aspergillus fumigatus copper export machinery and reactive oxygen intermediate defense counter host copper-mediated oxidative antimicrobial offense

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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 intrinsically 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 (AMφs), and reduced virulence in a non-neutropenic mouse. ΔaceA survival is remediated by dampening of host ROI (chemically or genetically) or enhancement of copper-exporting activity (CrpA) 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 (AMφs) 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 AMφs and neutrophils. AMφs reside beneath the alveolar surfactant film where they represent 90% of the resident leucocytes in the lung (Hasenberg et al., 2011). Molecular mechanisms by which AMφs 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; Kasahara 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-Santamarina 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 Ctr1 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). Deletion of either ctrA1 or ctrA2 results in attenuated virulence of C. neoformans. The copper transporter ctr4 in C. neoformans is essential for establishing full virulence during meningocencephalitis 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...
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-neutropenic IA model. Our biochemical and virulence data strongly support a mechanism of an inability of ΔaceA mutants to manage host-derived copper imported by host copper ion transporters. This macrophage sensitivity is corrected by tants to manage host-derived copper imported by host copper dynamics potentiate ROI stress for A. fumigatus: either ΔaceA regain of activity of the putative copper exporter CpaA 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 ΔaceA 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 ΔlueA 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 Theile, 1989). A domain search in the A. fumigatus At293 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 putative copper-binding transcription factors including Mac1p from S. cerevisiae and is most closely related to GRiSEA 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 close to CuF1 from C. neoformans (Figure S1A) (Ding et al., 2011). Yeast copper-binding transcription factors involved in copper detoxification including Crf1, Amt1, Cup2, and Cup2p/Arp1 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 pathways protecting from 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 (O$_2^-$) as a cofactor of copper-dependent SODs and on the other hand can contribute to hydroxyl radical (‘OH) production from hydrogen peroxide (H$_2$O$_2$) by participation in Fenton chemistry, we tested the mutants for synergistic effects of increasing copper and the intracellular O$_2^-$ 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 H$_2$O$_2$ 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 1-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 ΔmacA strain displayed a survival rate of ~25%, whereas the ΔaceA mutant only showed ~10% survival (Figure 2A). Interestingly, deletion of 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 ΔaceA 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 ΔaceA 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 S3A 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 ΔmacA mutant showed wild-type-like virulence; however, despite the elevated survival rate in the macrophage assay, the ΔcufA strain did not show increased virulence in this model (Figure 2C). Although not significant compared to wild type, analysis of the ΔaceA 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 ΔcufA or ΔmacA and wild type in this model (Figure 2E).

**Macrophage Copper Flux Is Altered in ΔaceA 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. 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. fumigatus* infection and measurable in copper levels in macrophage confrontations between wild-type and ΔaceA *A. fumigatus* strains.

We first examined for any alterations in ATP7A or Ctr1 dynamics. Western blot analysis of non-infected and *A. 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 3A; Figures S3D and S3E). Immunohistochemistry analysis of the murine copper ATPase ATP7A showed an increased fluorescent signal in *A. 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. fumigatus* spores (wild-type, ΔaceA, and aceAC 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. fumigatus* strains. As demonstrated with AMFs (Figure 2), the ΔaceA 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 \( \Delta \text{aceA} \) spores compared to unchallenged \( \Delta \text{aceA} \) spores (Figure 3D). This increase did not occur in wild-type and reconstituted \( \text{aceA} \) strains (Figure 3D). Quantification of the copper content in macrophages challenged with the different strains showed that the cells incubated with \( \Delta \text{aceA} \) spores had a decreased total copper concentration, suggesting a mobilization of copper to the \( \Delta \text{aceA} \) spores—a trend that was not observed for the wild type or the reconstituted \( \text{aceA} \) strain (Figure 3E). Importantly, the level of zinc or iron in macrophages incubated with the \( \Delta \text{aceA} \) spores was not decreased relative to the wild-type and \( \text{aceA}^{\text{C}} \) 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 \( \Delta \text{aceA} \) strains, we performed three experiments. First, we used the pharmacological PHOX complex inhibitor diphenyleneiodonium (DPI) (O’Donnell et al., 1993; Philippe et al., 2003) in our AM\( \Phi \) experiment and observed that survival of the \( \Delta \text{aceA} \) strain returned to

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**Figure 2. Deletion of \text{aceA} Reduces Fungal Survival and Virulence in Immunocompromised Mice**

(A) CFUs of fungal strains after incubation with murine AM\( \Phi \)s for 2 hr. Experiments were carried out in triplicates; error bars represent SDs, and statistical significance is indicated by \( p \) values.

(B) Survival rates of mice immunocompromised with cortisone acetate and infected with the \( \text{A. fumigatus} \) wild type, \( \Delta \text{aceA} \), and the reconstituted strain \( \text{aceA}^{\text{C}} \), respectively. Statistical significance is indicated by \( p \) values. Ten mice were in each group.

(C) Survival rates of mice immunocompromised with cortisone acetate and infected with the \( \text{A. fumigatus} \) wild-type, \( \Delta \text{macA} \), and \( \Delta \text{cufA} \) strains, respectively. Statistical significance is indicated by \( p \) values.

(D) Survival rates of mice immunocompromised with cyclophosphamide and infected with the \( \text{A. fumigatus} \) wild-type, \( \Delta \text{aceA} \), and the reconstituted strain \( \text{aceA}^{\text{C}} \), respectively. Ten mice were in each group.

(E) Survival rates of mice immunocompromised with cyclophosphamide and infected with the \( \text{A. fumigatus} \) wild-type, \( \Delta \text{macA} \), and \( \Delta \text{cufA} \) strains, respectively.

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**Depleting Host ROI Synthesis Remediates \( \Delta \text{aceA} \) Survival in Host Tissues**

Considering that the \( \Delta \text{aceA} \) strain grew poorly in the ATP7A-deficient larval zebrafish and is sensitive to ROI, we considered an alternative host mechanism in addressing the \( \Delta \text{aceA} \) phenotype. Because macrophages deploy mechanisms of \( \text{O}_2^- \) 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 \( \Delta \text{aceA} \) strain, we asked whether dampening ROI stress would restore \( \Delta \text{aceA} \) survival in AM\( \Phi \)s.

To test whether inhibition of \( \text{O}_2^- \) production by host immune cells and/or copper limitation by chemical chelation would restore wild-type-like survival rates of the \( \Delta \text{aceA} \) mutant, we performed three experiments. First, we used the pharmacological PHOX complex inhibitor diphenyleneiodonium (DPI) (O’Donnell et al., 1993; Philippe et al., 2003) in our AM\( \Phi \) experiment and observed that survival of the \( \Delta \text{aceA} \) 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 ΔaceA persistence in whole-larval homogenates revealed that attenuated ΔaceA survival was dependent on p22phox expression (Figure 4B). Third, we compared fungal burden of the A. fumigatus wild type and the ΔaceA 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
both wild type and ∆aceA in copper-depleted and excess conditions. We tested expression of the four copper importers identified in A. fumigatus (Table 1) as well as genes implicated in copper detoxification. Search of the A. fumigatus genome for putative homologs of the C. albicans copper exporter-encoding gene crp1 and the S. cerevisiae copper metallothioneins CUP1 and CRS5 identified one homolog each that we call crpA (G12740) and cmtA (AfuA 4G04318), respectively (Table 1; Figure S4A). We also tested for the expression of the copper-dependent SOD sod1 and the two mycelial catalases cat1 and cat2 as well as the spore catalase catA.

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

The Putative Copper-Exporting P-Type ATPase CrpA and Spore-Specific ROI Defense bZIP Transcription Factor AtfA Mediate ∆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 ∆aceA phenotype. To test the former hypothesis, we investigated whether constitutive expression of the A. fumigatus bZIP transcription factor-encoding gene atfA, which is known for its involvement in spore maturation and spore ROI defense (Hagiwara et al., 2009, 2014, 2016), could restore the ∆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 ∆atfA mutant compared to the wild type that was similar to the ∆aceA strain (Figure 5B). Forced expression of atfA brought survival back to wild-type levels in a ∆aceA background (Figure 5B) despite its poor growth phenotype when grown on solidified media (Figure S5A). Because AtfA is suggested to specifically govern spore ROI defense, we tested spore sensitivity toward H2O2 with 5 μM 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 AMs (Figures S5C and S5D). In contrast, deletion of crpA resulted in hypersensitivity to copper compared to the wild type and ΔaceA strain (Figure 5C). 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 S3H). 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 S3H).

**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 phagocytic 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 TTM 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 reoccuring urinary tract infections (Türner 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., 2016), whereas the copper importers Ctrl/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; Novoa-Aponte and Soto Ospina, 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 pyR 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 x 10⁶ 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 x 10⁶ 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 x 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 α32P.

**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 quantitated 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 (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 x 10^5 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 x 10^7 cells/well) and killing assays (1 x 10^6 cells/well), spores were incubated with cells in a 3:1 (spore:cell ratio) plus indicated supplements to complete AMφ- media. Cells and spores were centrifuged at 300 x 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 150 mg/kg cortisone acetate 3 days prior to infection, and with cyclophosphamide (150 mg/kg) on the day of their infection, 3, 7, and 11 days post-infection. In the neutropenic (cyclophosphamide) model, mice were injected subcutaneously with cortisone acetate (300 mg/kg) 3 days prior to infection, on the day of their infection, and with cyclophosphamide (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 x 10^6 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).

**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 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 injection. 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.

**Larval Zebrafish Infection Model**

All larval zebrafish infection experiments were performed as described (Knox et al., 2014). Morpholino-mediated genetic knockdown of p22phox or atp7a was obtained as previously described (Tauzin et al., 2014). Immediately following microinjection, g–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 http://dx.doi.org/10.1016/j.celrep.2017.04.019.

**AUTHOR CONTRIBUTIONS**


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