Supplemental Experimental Procedures

Fungal strains and culture conditions

A. 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 $ZnSO_4$ x 7 H_2O, 11 g/LH_3BO_3, 5 g/L $MnCl_2$ x 4 H_2O, 1.6 g/L $FeSO_4$ x 7 H_2O, 1.6 g/L $CoCl_2$ x 5 H_2O, and 1.1 g/L $(NH_4)_6Mo_7O_{24}$ x 4H_2O. To adjust for different Cu conditions, Cu was added from a 100 mM $CuSO_4$ 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 10^6 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 10^8 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 cm^2 disks punched out of the plates after 5 days of incubation at 37 °C in the dark. For harvesting spores for macrophage survival assays, all strains were grown for 3 days at 37 °C in the dark on GMM + 1 µM Cu to ensure comparable growth and melanization of spores. Spores were harvested in PBS (Cornig Cellgro; 21-030-CV) and passed through a cell strainer before performing the assay. For colony forming unit enumeration, spores were plated on GMM + 1 µM Cu and incubated for 2 days at 37 °C in the dark.

For zebrafish larvae infection experiments special care was taken in preparation for spore microinjection to minimize clumping in the glass capillary needle. Fungal strains were inoculated onto GMM plates at a concentration of 1 x 10^6 conidia per plate using an overlay method and grown for 3 days at 37 °C. Conidia 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 10^8 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_prom_F/pyrG_term_R (Table S2). The primers [gene]-5R and [gene]-3F contain complement sequences to the primers pyrG_prom_F 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/OE:crpA-R. Transformation was performed as previously described (Palmer et al., 2008). For selection of Δ*aceA* transformants, Cu and uracil/uridine were omitted in the selection media. For selection of Δ*macA* 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 Δ*aceA*/OE::*atf1* we first created a Δ*argB* strain from CEA17 KU80 as described for the other gene deletions above. Transformants were selected on media containing 1 g/L arginine. Subsequently we transformaned ΔargB with a construct that had the two pyrG flanks fused to each other using the primer pair XX. Protoplast were selected on media containing 5mM uracil/uridine, 1 g/L arginine and 1 g/L 5-fluoro-orotic acid (5-FOA) as previously described (d'Enfert, 1996) creating strain ΔΔ*pyrG*/*argB*. This strain was used to delete Δ*aceA* as described above creating strain ΔΔ*argB*/*aceA*. For overexpression of atf1, a *gpdA* promoter/*argB*/5'*atfA* fusion construct was created using primer pairs 60/61 and 62/63 and argBF (5'-gcttgaagtattatgggat)/gpdR (5'-catggtgatgtctgctcaagcgggg) (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 P^{32}-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 αP^{32}.

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 quantifies using an Epoch2 microplate reader (BioTek) and equal amounts were reconstituted in 2x LDS buffer [0.5 M Tris/HCl (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 Ctr1 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 Ctr1 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 followed by incubation at room temperature with Alexa Fluor 488 goat anti-rabbit antibodies (1:1000) in TBS-T containing 0.1% non-fat milk for 1 h at room temperature and washed in TBS-T as described above.

Coverslips were mounted onto a pre-cleaned microscope slide. Images were taken with a Zeiss AxioImager A10 equipped with a Zeiss EC Plan-NEOFLUAR 100x/1.3 Oil DIC/∞/0.17 objective and a series 120X-Cite® light source (EXFO).

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.

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 (Subramanian 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 $1x10^5$ cells/well and allowed to rest overnight in a 37ºC humidified incubator (5% CO_2) 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% CO_2) 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 were prepared by culturing L929 fibroblast cells in DMEM supplemented with 10% heat-inactivated FBS, 1% GlutaMAX™ (Life Technologies; 35050), and 1% pen/strep for ten days in a 37ºC humidified incubator (5% CO_2). The L929-conditioned medium was sterile-filtered through a 0.22 µM filter and stored at -80ºC until ready for use.

Murine alveolar and bone marrow derived macrophage killing assays

For metal quantification (1×10^7 cells/well) and killing assays (1×10^5 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 5×10^5 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 littermates 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′ GGCCCTTAAATAGCCCGGT-3′ AF 18S rev: 5′-TGAGCCGATAGTCCCCCTAA-3′ AF 18S probe: 5′-FAM-AGCCAGCGGCCCGCAAATG- 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 Δ*aceA* 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 $p22^{phox}$ or atp7a was obtained as previously described (Tauzin 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 10^8 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 H_2O_2 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.

Supplemental Figures

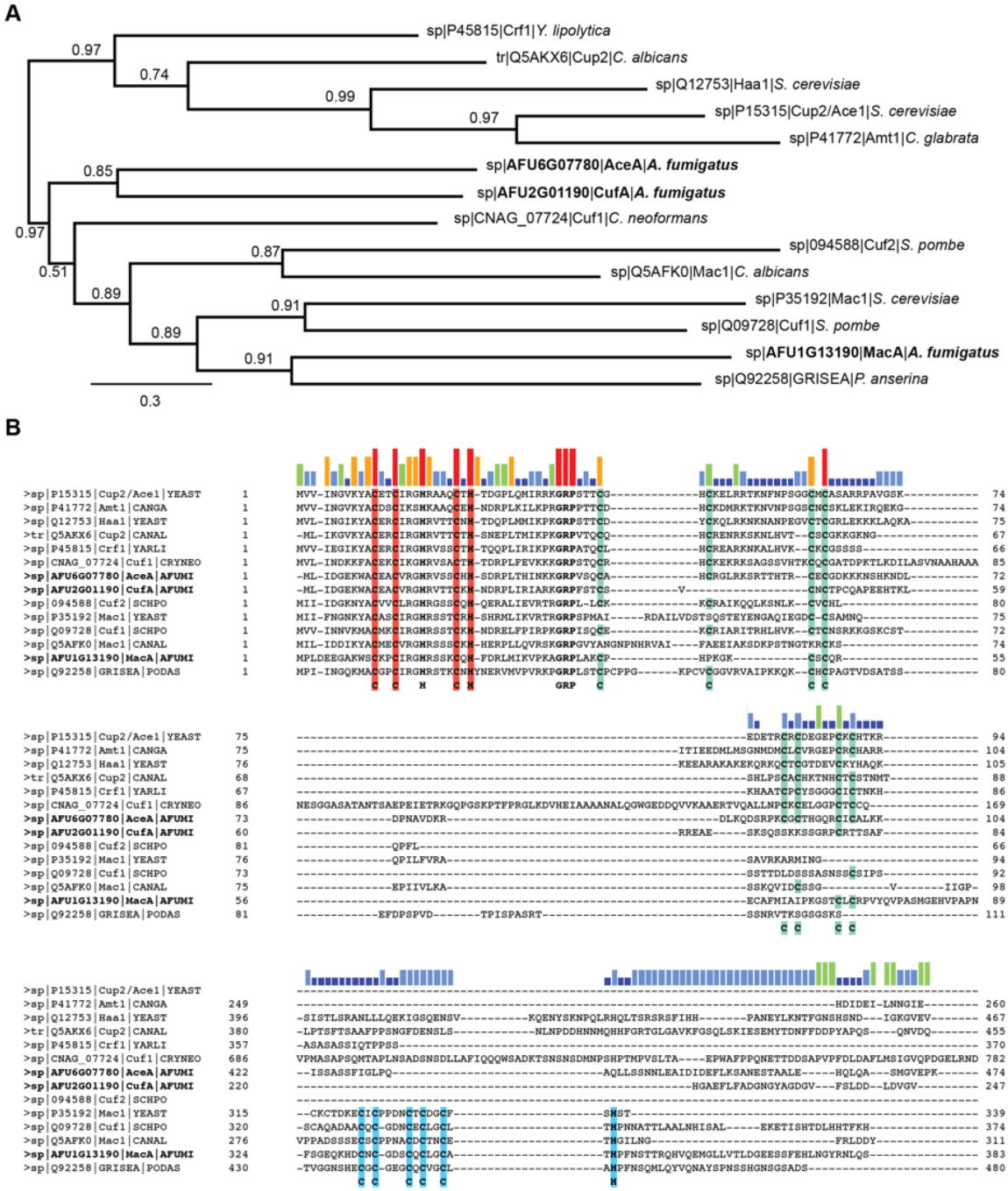

Figure S1. Phylogenetic relation and domain architecture of Cu-Fist transcription factors. Related to Table 1.

(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.

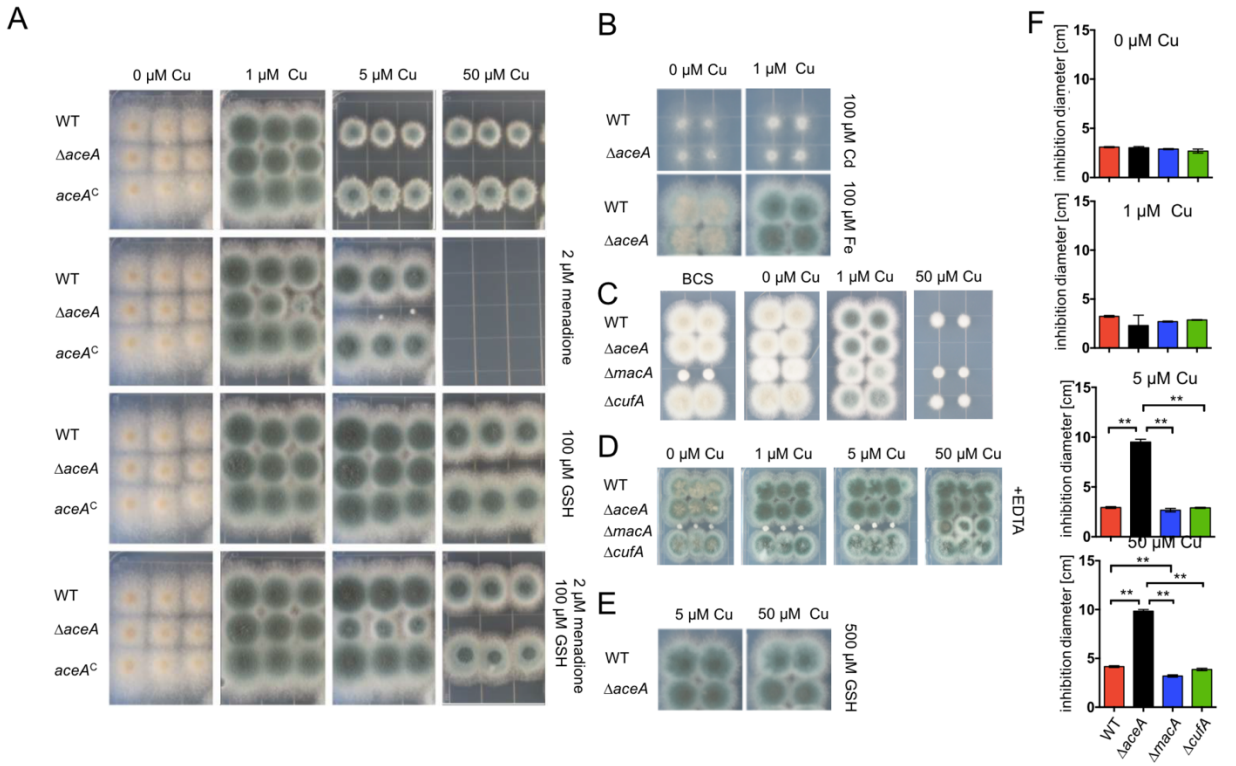

Figure S2. Growth phenotypes of the Cu-Fist mutants on different metal stresses. Related to Figure 1.

(A) 2000 spores of indicated strains grown on solidified glucose minimal medium (GMM) without EDTA with indicated concentration of $CuSO_4$ for 72 h at 37°C.

(B) 2000 spores of indicated strains grown on solidified GMM with reduced Cu concentrations amended with 100 µM $CdSO_4$ and Fe SO_4, 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 $CuSO_4$ 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) H_2O_2 according to Thön et al., 2007. Experiment was carried out in triplicate, error bars represent standard deviations and asterisks indicate statistical difference, $p < 0.05$.

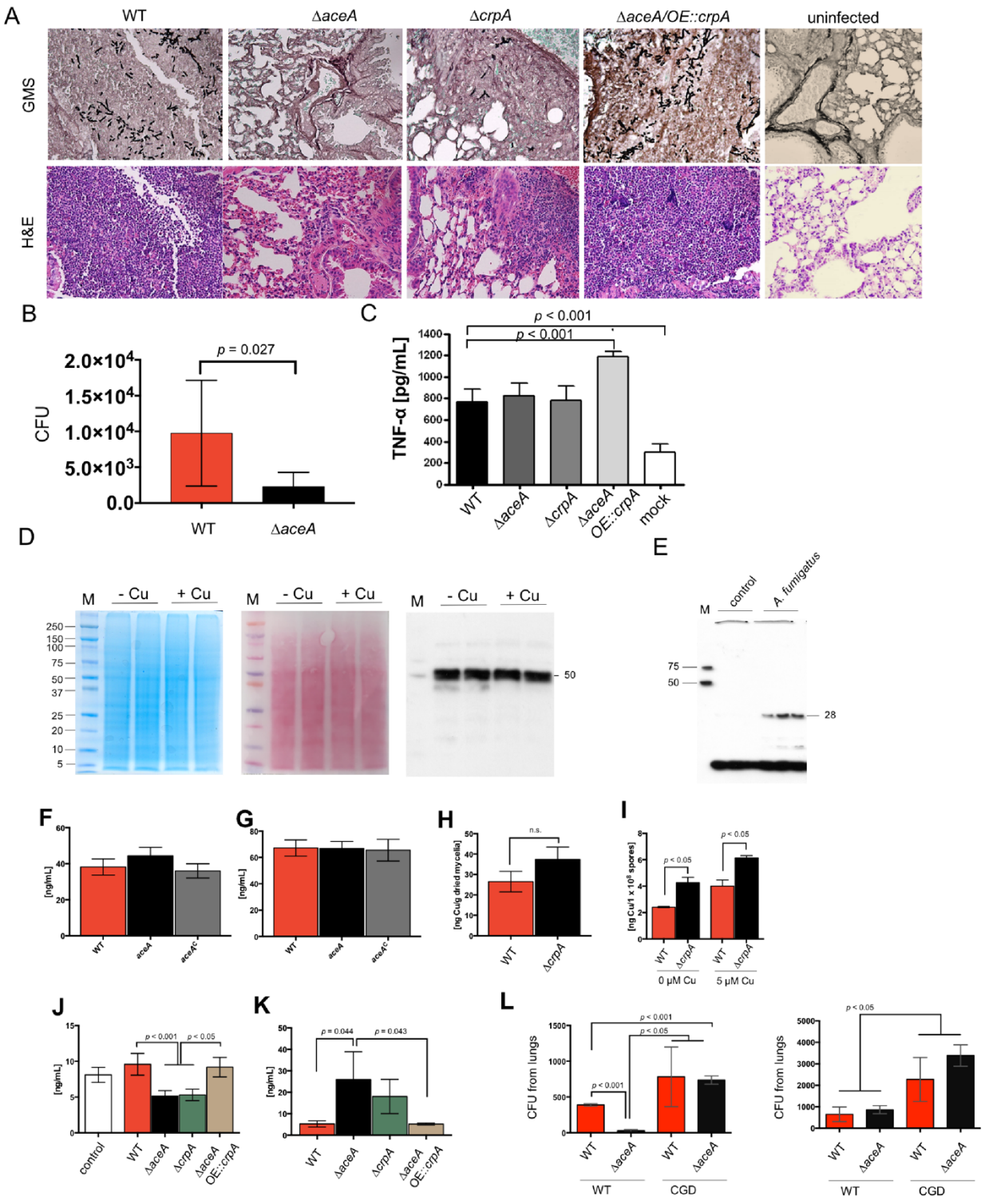

Figure S3: Inflammatory responses, fungal survival and histopathology, and metal quantification of wild-type and Δ*aceA* infected mice from our infection models. Related to Figure 2, Figure 3, Figure 4 and Figure 5.

(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 Δ*aceA* 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 shoes 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.

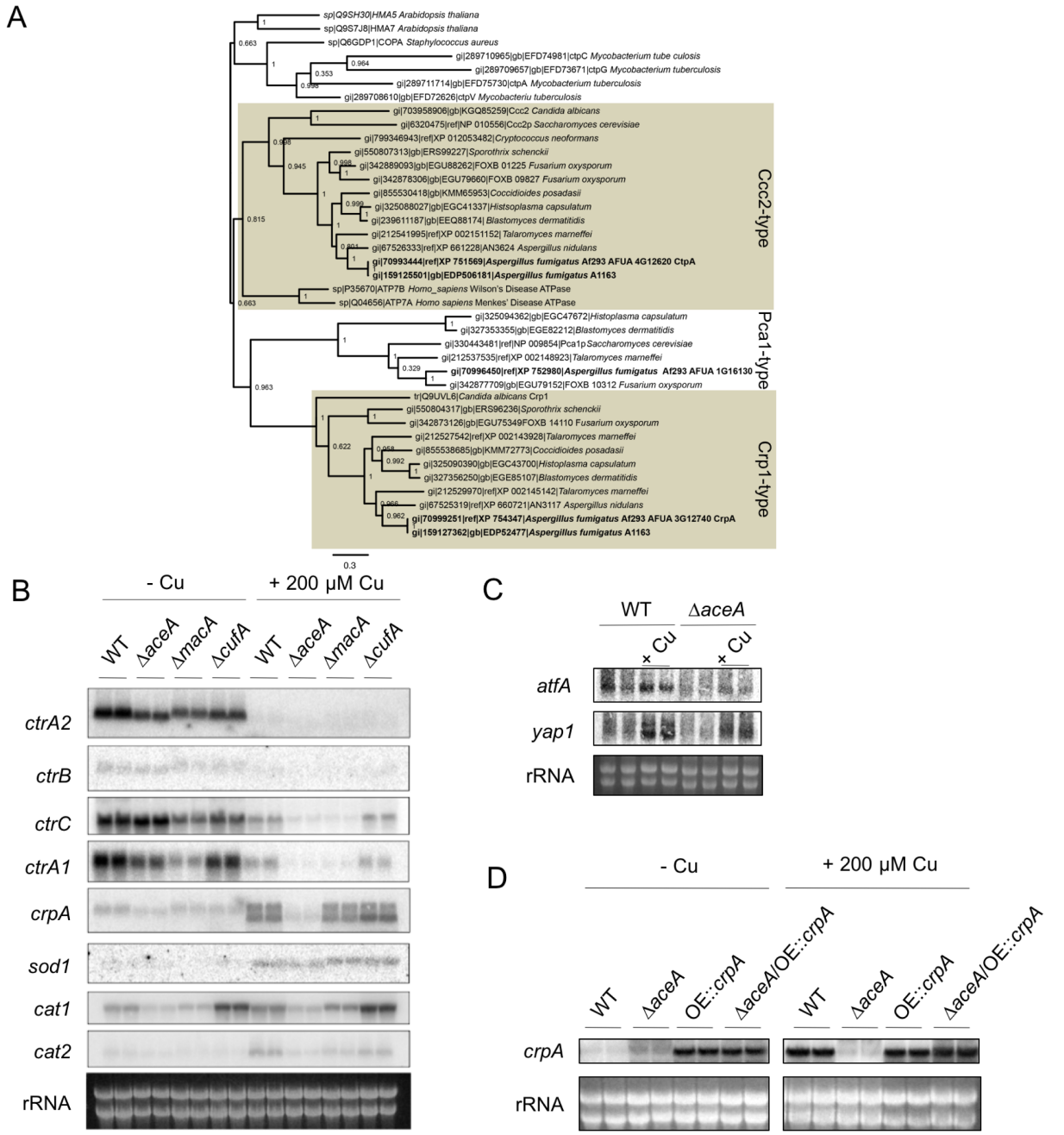

Figure S4: Phylogenetic relation and domain architecture of heavy metal ATPases (HMA) of select species and Cu-dependent expression analysis. Related to Figure 5.

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.

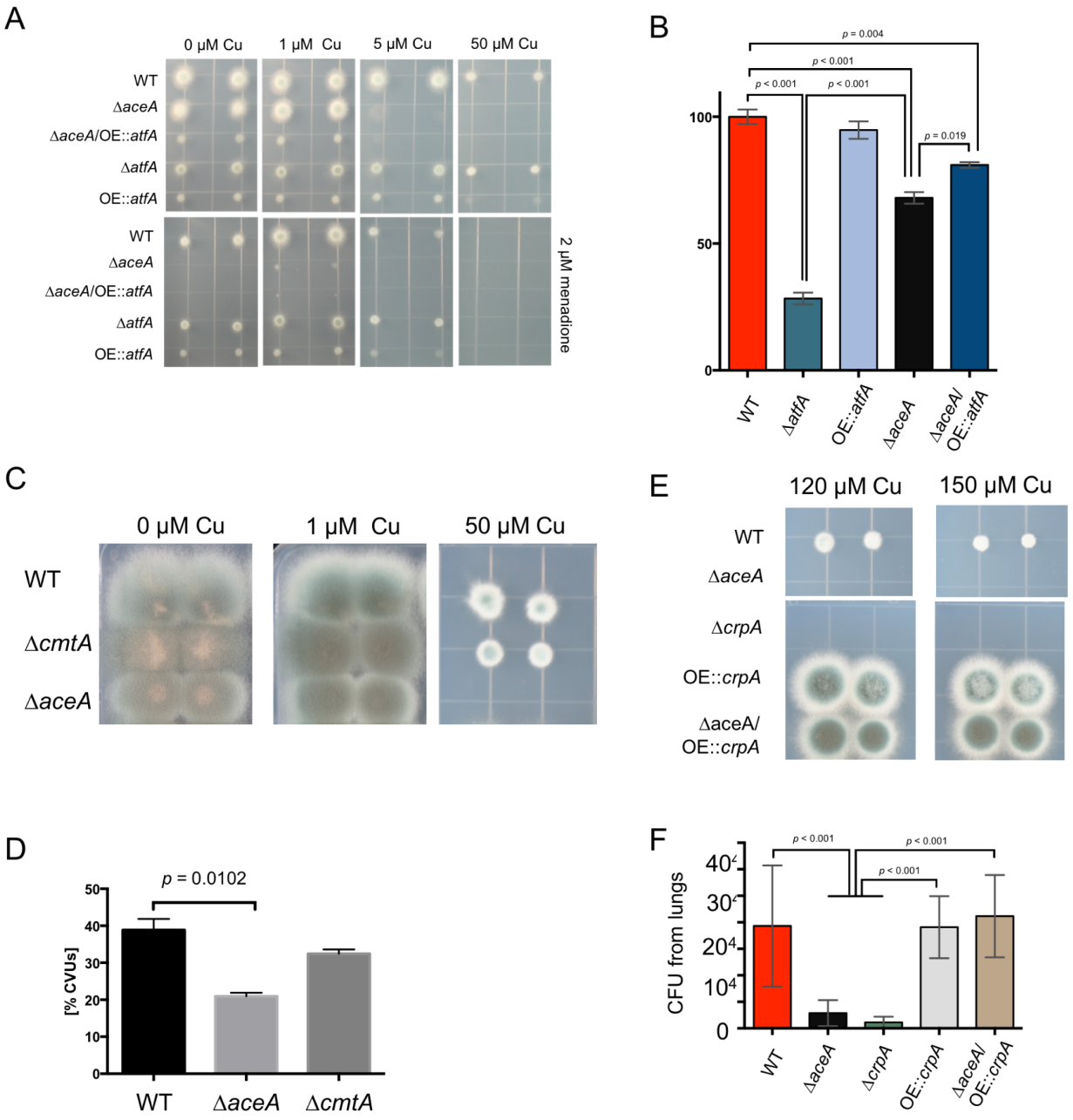

Fig. S5: Characterization of the metallothionein CmtA in *A. fumigatus* and growth phenotypes of *crpA* and *atfA* over-expression strains on elevated Cu concentrations. Related to Figure 5.

(A) 2000 spores of indicated strains grown on solidified glucose minimal medium (GMM) without EDTA with indicated concentration of $CuSO_4$ and menadione for 48 h at 37°C.

(B) CFU of spores incubated in GMM supplemented with 5 µM Cu and 10 mM H_2O_2 for 1 h at 37 °C. After incubation spores were washed twice and plated in serial dilutions on GMM containing 1µM 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 $CuSO_4$ 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 $CuSO_4$ 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.

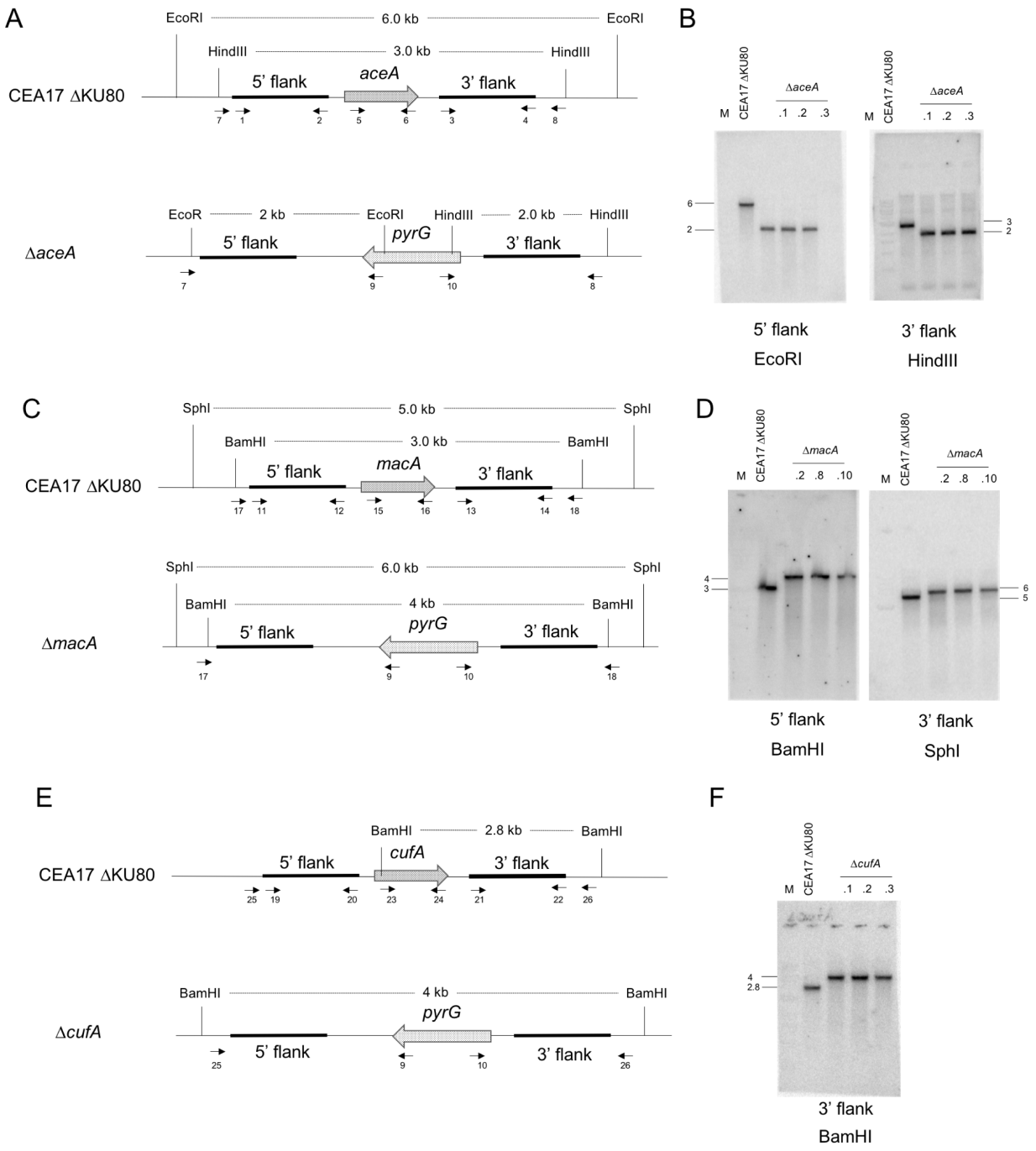

Figure S6: Deletion strategy and Southern analysis of Δ*aceA*, Δ*macA* and Δ*cufA* strains. Related to Experimental Procedures.

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

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

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

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

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

(F) Southern blot analysis of recipient strain and Δ*cufA* strains. Restriction was carried out using the indicated

enzymes and probed with the indicated PCR fragments.

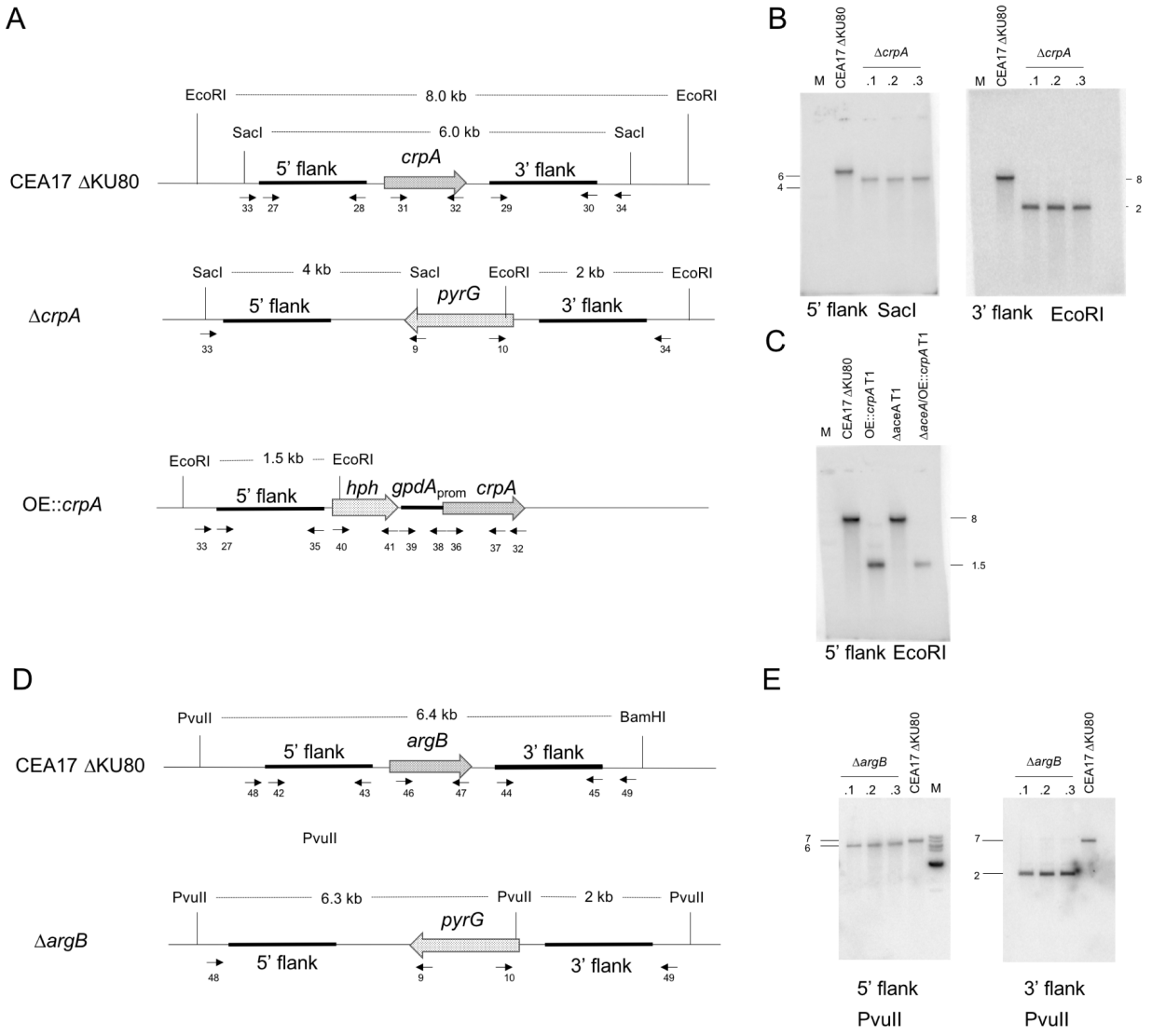

Figure S7: Deletion strategy and Southern analysis of Δ*crpA*, OE::*crpA* and Δ*argB* strains. Related to Experimental Procedures.

(A) Deletion strategy for Δ*macA* and OE::*crpA* strains. Arrows indicate positions of primers used (Table S2).
(B) Southern blot analysis of recipient strain and Δ*crpA* strains. Restriction was carried out using the indicated enzymes and probed with the indicated PCR fragments.
(C) Southern blot analysis of recipient straisn and OE::*crpA* strains. Restriction was carried out using the indicated enzymes and probed with the indicated PCR fragments
(D) Deletion strategy for Δ*argB* strains. Arrows indicate positions of primers used (Table S2).
(E) Southern blot analysis of recipient strain and Δ*argB* strains. Restriction was carried out using the indicated enzymes and probed with the indicated PCR fragments.

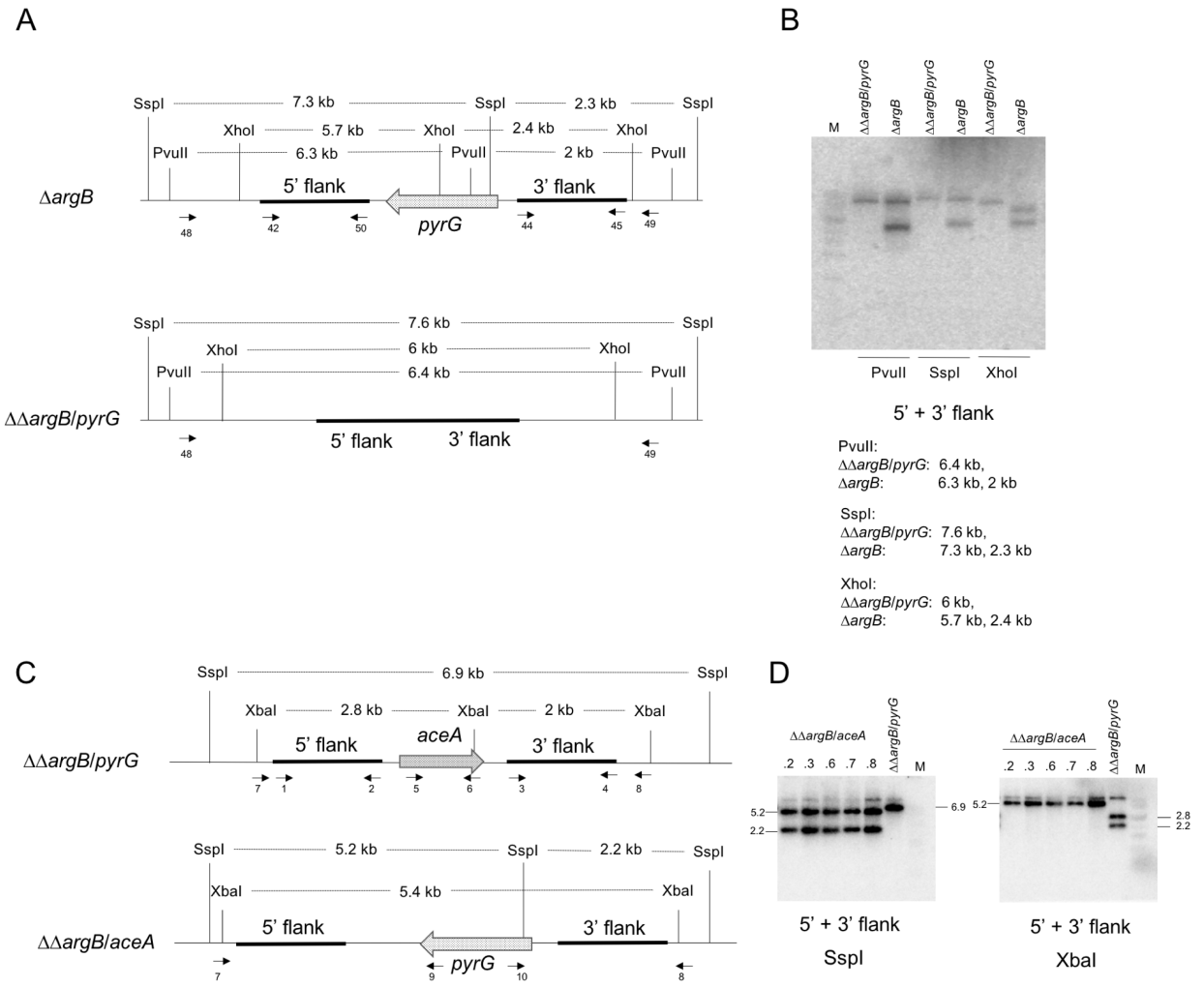

Figure S8: Deletion strategy and Southern analysis of ΔΔ*argB*/*pyrG* and ΔΔ*argB*/*aceA* strains. Related to Experimental Procedures.

(A) Deletion strategy for ΔΔ*argB*/*pyrG* strains. Arrows indicate positions of primers used (Table S2).

(B) Southern blot analysis of recipient strain and ΔΔ*argB*/*pyrG* strains. Restriction was carried out using the indicated enzymes and probed with the indicated PCR fragments.

(C) Deletion strategy for ΔΔ*argB*/*aceA* strains. Arrows indicate positions of primers used (Table S2).

(D) Southern blot analysis of recipient strain and ΔΔ*argB*/*aceA* strains. Restriction was carried out using the indicated enzymes and probed with the indicated PCR fragments.

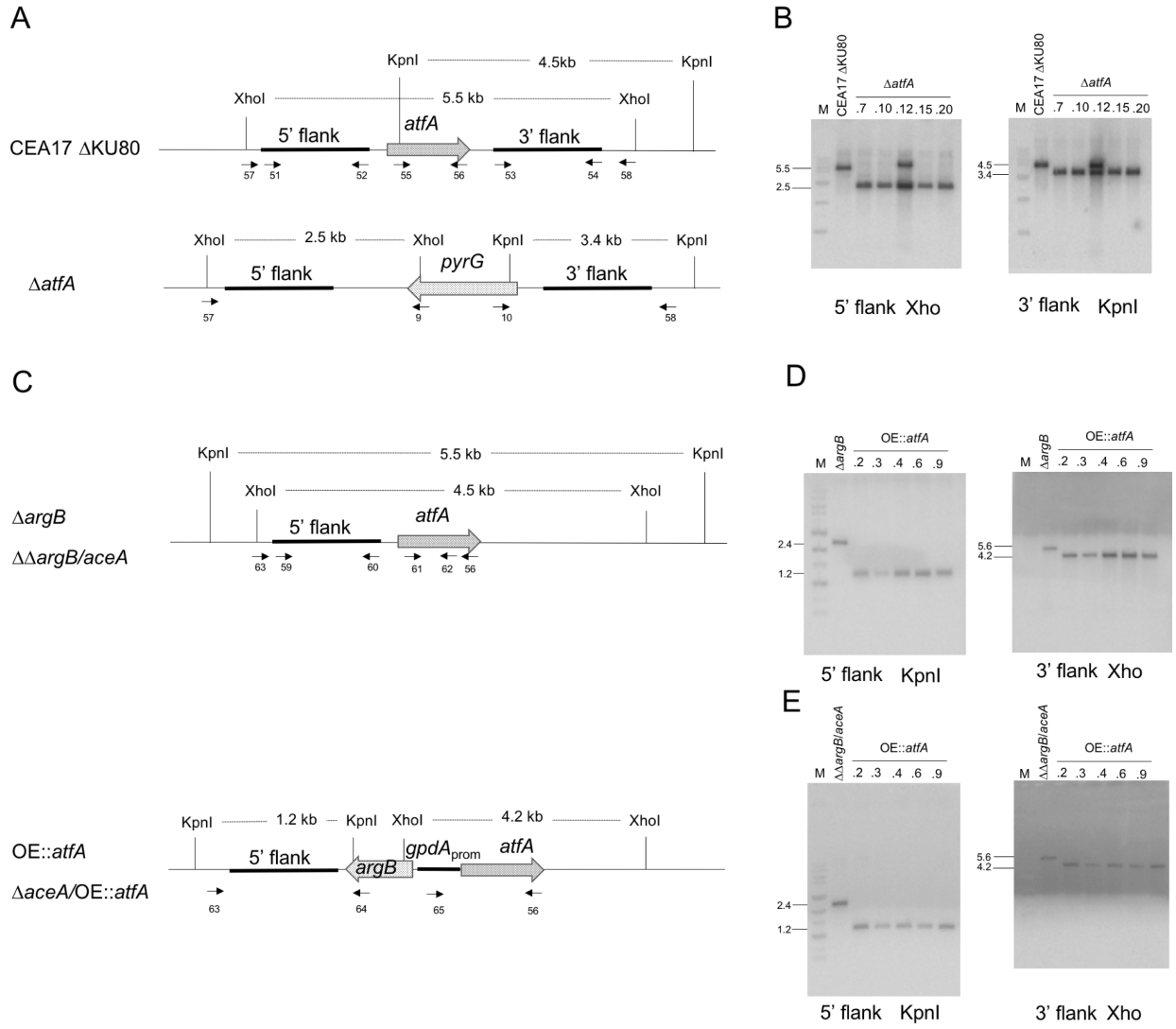

Figure S9: Deletion strategy and Southern analysis of Δ*atfA* OE::*atfA* and Δ*aceA*/OE::*atfA* strains. Related to Experimental Procedures.

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

B) Southern blot analysis of recipient strain and Δ*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 Δ*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 ΔΔ*argB*/*aceA* and Δ*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.

strain	genotype	reference
CEA17*pyrG*+	*pyrG1*, Δ*akuB*::*pyrG*	da Silva Ferreira et al., 2006
CEA17 KU80	*pyrG1,* Δ*akuB*::*pyrG*, *pyrG1*	da Silva Ferreira et al., 2006
Δ*aceA*	*pyrG1,* Δ*akuB*::*pyrG*, *pyrG1*, Δ*AFUB_073740*::*pyrG*	this study
aceA^{C}	*pyrG1,* Δ*akuB*::*pyrG*, *pyrG1*, Δ*AFUB_073740*::*pyrG, AFUB_073740*::*hph*	this study
Δ*macA*	*pyrG1,* Δ*akuB*::*pyrG*, *pyrG1*, Δ*AFUB_012670*::*pyrG*	this study
Δ*cufA*	*pyrG1,* Δ*akuB*::*pyrG*, *pyrG1*, Δ*AFUB_018270*::*pyrG*	this study
Δ*crpA*	*pyrG1,* Δ*akuB*::*pyrG*, *pyrG1*, Δ*AFUB_036430*::*pyrG*	this study
Δ*cmtA*	*pyrG1,* Δ*akuB*::*pyrG*, *pyrG1*, Δ*AFUB_098700*::*pyrG*	this study
OE::*crpA*	*pyrG1,* Δ*akuB*::*pyrG*, *gpdA*(p)::*AFUB_036430*::*hyg*	this study
Δ*aceA*/OE::*crpA*	*pyrG1,* ΔakuB::pyrG, pyrG1, Δ*AFUB_073740*::pyrG, *gpdA*(p)::*AFUB_036430*::*hyg*	this study
Δ*argB*	*pyrG1,* Δ*akuB*::*pyrG*, *pyrG1*, Δ*AFUB_064280*::*pyrG*	this study
ΔΔ*argB*/*pyrG*	*pyrG1,* Δ*akuB*::*pyrG*, *pyrG1*, Δ*AFUB_064280*::*pyrG*, Δ*pyrG*	this study
ΔΔ*argB*/*aceA*	*pyrG1,* Δ*akuB*::*pyrG*, *pyrG1*, Δ*AFUB_064280*::*pyrG*, Δ*pyrG*, Δ*AFUB_073740*::*pyrG*	this study
Δ*atfA*	*pyrG1,* Δ*akuB*::*pyrG*, *pyrG1*, Δ*AFUB_037850*::*pyrG*	this study
OE::*atfA*	*pyrG1,* Δ*akuB*::*pyrG*, *pyrG1*, Δ*AFUB_064280*::*pyrG*, *gpdA*(p)::*AFUB_037850*::*argB*	this study
Δ*aceA*/OE::*atfA*	*pyrG1,* Δ*akuB*::*pyrG*, *pyrG1*, Δ*AFUB_064280*::*pyrG*, Δ*pyrG*, Δ*AFUB_073740*::*pyrG*, *gpdA*(p)::*AFUB_037850*::*argB*	this study

Table S2: Primers used in this study. Related to Experimental Procedures.

number	name	Sequence 5'-3'
1	PW_AceA_5F	CGTGGTCTTGCCGGTCAACGG
2	PW_AceA_5R	cccaattcgccctatagtgagtcgtattacgGAACAGGAGGCCCTGTCGGG
3	PW_AceA_3F	ggctgtcgctgcagcctctccgattgtcgaatCTGAGTGACGACCTCCGCCC
4	PW_AceA_3R	CCACCCACATGCGAACCGGC
5	PW_AceA-F	GCTGCACTCATGGTCAGCGC
6	PW_AceA-R	GGCAGTCGACTCGTTGGCGG
7	PW_AceA diag 5'	CACTACGTCCGATGTTCCGCG
8	PW_AceA_diag 3'	CCACCCACATGCGAACCGGC
9	PW pyrG diag 3'	ctggctataccgcccgaggc
10	PW pyrG diag 5'	gataagtccaaccttatcggcc
11	PW_MacA_5F	GATGACGGAGGATGGGAGAGG
12	PW_MacA_5R	cccaattcgccctatagtgagtcgtattacgGACCATATGTTGACTGTCGACC
13	PW_MacA_3F	ggctgtcgctgcagcctctccgattgtcgaatCCGTTCAACTACAACTTGACC
14	PW_MacA_3R	CATGTACGATACCTGCATAGG
16	PW_MacA_F	CCCAACTGCAACCATGCC
17	PW_MacA_R	GATTCCTCACCATCAAGCG
18	PW_MacA_5' diag	CCTCCGTAGCCTGGACGG
19	PW_MacA_3' diag	CGGAGCTGGTCGTGCCTGG
20	PW_CufA_5R	cccaattcgccctatagtgagtcgtattacgCCGGAGATCGTCTGGTCTTGC
21	PW_CufA_3F	ggctgtcgctgcagcctctccgattgtcgaatgggatttgtcattgcccatgc
22	PW_CufA_3R	GATAGGGTTACCGTCATTAAGC
23	PW_CufA_F	GCTTGCGTCCGCGGCCACC
24	PW_CufA_R	GGACTGGGAGTGCTGGGG
25	PW_CufA_5' diag	GCTGCGACATGGTAGATTGG
26	PW_CufA_3' diag	CCGGTGCCTATTCCTACCGG
27	PW crpA 5F	ggcccagtgggatagtcacgtg
28	PW crpA KO 5R	cccaattcgccctatagtgagtcgtattacggcaagaccagtcagccaggatctc
29	PW crpA 3F	cggctgtcgctgcagcctctccgattgtcgaatCGGTCTATGTCTTGAaggagttgg
30	PW crpA 3R	gcccaagaatccctgaatggc
31	PW_CrpA_F	GATGGCCTTGGTTACGAGGCCTCCC
32	PW_CrpA_R	CCCAGCCACCCCGTGCCGTGTCAAGG
33	PW crpA diag 5	catcgaagagccactagcag
34	PW crpA diag 3	gactcgagtactcatgtccgg
35	PW crpA OE 5R	ctgtcttctactataacttcctcgtattacgctctcccgagaatcgaaccgcc
36	PW crpA OE F	cttgagcagacatcaccatgATGGCTACGGAAACGAGGCCTC
37	PW crpA OE R	CGACCACAGGTTGGATGTTGTCG
38	PW gpdA prom R	atccacttaacgttactgaaatccatggtgatgtctgctcaag
39	PW_gpdA prom F	aattccatccggatgtcgaaggc
40	hphF-trpC-P	gacagaagatgatattgaaggagc
41	hphR-trpC-T	gatttcagtaacgttaagtggat
42	PW argB 5F	gtgagtttgaatactgccgc
43	PW argB 5R	cccaattcgccctatagtgagtcgtattacgGTTGGCCGTTCAATGCGCCG
44	PW argB 3F	ggctgtcgctgcagcctctccgattgtcgaatTTCCCTGAAGCAGAGAATCG
45	PW argB 3R	gtgcgtcgtcctggctctgc
46	PW argB F	GTGCGGATGGGAGGCCACC
47	PW argB R	GTCCTTGAGCCGCTTAATCG
48	PW argB 5' diag	ctggttcgcacgccatcc
49	PW argB 3' diag	gggctgatgccgcgatgagg
50	PW argB 5R pyrG	CGATTCTCTGCTTCAGGGAAGTTGGCCGTTCAATGCGCCG
51	PW argB 3F pyrG	TTCCCTGAAGCAGAGAATCG
52	PW atfA 5F	GCAAGCATTCTTTTACCGTTCTCTCC
53	PW atfA 5R	cccaattcgccctatagtgagtcgtattacgGGAGGGTAGCCGATGAGAGTGACC
54	PW atfA 3F	ggctgtcgctgcagcctctccgattgtcgaaCCGCTCATGATCTTTAATTGACGCC

55	PW atfA 3R	CTCGAGTCAATCGTGTGTGACGCGG
56	PW atfA F	CCGCCTTTACAACACGCAC
57	PW atfA R	CGCCTGAAGATTGGCAAGCC
58	PW atfA 5’ diag	CTTCTATGCTGCTTCTGGACTCTCG
59	PW atfA 3’ diag	CTCGATTGCTGGCAATTTCTCGAGG
60	AS atfA OE 5F	CTAAGCCTGGCATAATGCATGC
61	AS atfA OE 3R	cgccctatagtgagtcgtattacgCCCTGTTTGGTTGAGTAATCTGG
62	AS atfA OE 3F	ccccgcttgagcagacatcaccatgATGTCTGCTGCTGTCGCTTCG
63	AS atfA OE 3R	CGTTCTTCTTGGCAGCGCTCAA
64	AS atfA OE 5’ diag	CACAACATGAATCATGCCAGG
65	AS argB 5’ diag	cctcacctacagagtacatc
66	AS gpdA 3’ diag	ccatactccatccttcccatc
67	PW_CmtA_F	GGTCCACCCGTCTTCCACC
68	PW_CmtA_R	GGCTTCAGCCGCCGCATCAAC
69	PW_CmtA_5F	CGCTAGCTTCGCTCCCGGTCC
70	PW_CmtA_5R	cccaattcgccctatagtgagtcgtattacgCCGAAGTGATGAGGAAGCTCGAGG
71	PW_CmtA_3F	ggctgtcgctgcagcctctccgattgtcgaatGCCGCATCACTACTGGTGGC
72	PW_gpdA_int_F	gaagggtggtgccaagaag
73	PW_gpdA_int_R	caacggagacgttggaggt
74	PW_CtrC-F	CCACAATATGGACTCCATGG
75	PW_CtrC-R	GCTCCTTGCATATCATCTGAGG
76	PW_SOD1-F	GGTGACTCCAAGATCACC
77	PW_SOD1-R	GCGTTACCAGTCTTCTTGG
78	PW_CtrA2-F	CCATTCGACCATGTCTTCC
79	PW_CtrA2-R	CGTTCCATTGGATGTACCG
80	PW_Cat1_F	CCTGGTCACATTGTCCGTGG
81	PW_Cat1_R	CATCAGTGACTGACTGAGCC
82	PW_SOD2_F	CTCATCCGTACCTCTGCGCG
83	PW_SOD2_R	GAAGCGCTTCTCCACAGCC
84	PW_SOD3_F	CACGCTCCCACCCCTCCCC
85	PW_SOD3_R	GTCCACCCTTGTCACCCGC
86	PW_CatA_F	CCCAGATCAGCCGCCTGGGC
87	PW_CatA_R	CCACAAAGTCCGTTGCGCCC
88	PW_Cat2_F	CGCCTGGTTCAAGCTCACCC
89	PW_Cat2_R	CAGGGATGGTCTGCTTGCCC
90	PW_CtrB_F	CTTCTCCCTCCTCATGTCCC
91	PW_CtrB_R	CAACTGCAAGCATGACGAACCC
92	PW CtrA1 F	CAATGGTCTTCGACACCAGCACCG
93	PW CtrA1 R	CCACTACAATGCCTCCCACCACCGC